치의과학 박사학위논문

Establishment of parthenogenetic murine embryonic stem cells and their differentiation into osteogenic and chondrogenic cells

마우스 단위발생 배아줄기세포주의 확립과
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강 호 인

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ABSTRACT

Establishment of parthenogenetic murine embryonic stem cells and their differentiation into osteogenic and chondrogenic cells

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Damaged tissue repair using stem cells has become a topic of great interest in tissue engineering including orthopedics and dentistry research. Embryonic stem cells (ESCs) are pluripotent and can differentiate into all somatic cell types. ESCs are an alternative solution to hard and soft osseous tissue regeneration and skeletal tissue repair to treat bone diseases and defects using regenerative strategies. The aim of this thesis was to improve the efficiency of stem cell generation and differentiation by modifying traditional methods. In this study, I used a new culture method called the oil-free micro-tube culture

method (MTC). This method was shown to be effective for the generation of murine parthenogenetic embryonic stem cell (PESCs) lines. Murine parthenogenetic embryos cultured in MTC showed higher rates of stem cell line generation than those in the traditional micro-drop culture method, and this may be due to the promoted expression of developmentally important genes such as *Igf1* and *Oct4*. PESCs may be a useful alternative stem cell source for tissue repair and regeneration. The defects in full-term development of this cell type enable researchers to avoid the ethical concerns related with ESC research. However, many previous reports have shown that the differentiation potential of PESCs is limited compared to that of ESCs, and this may be related to the abnormal expression of imprinting genes in PESCs. Hence, it is hypothesized that specific-imprinting gene expression promotes the osseous differentiation of PESCs. Insulin like growth factor *2* (IGF2) is a paternally expressed imprinting gene that is therefore not expressed in PESCs naturally. Osteogenic and chondrogenic differentiation of PESCs promoted by IGF2 was demonstrated. The lack of endogenous IGF2 expression can be compensated by exogenous supplementation of this soluble factor in the culture medium resulting in osteogenic and chondrogenic cell differentiation. In osteogenic cell differentiation, gene expression of specific osteoblastic markers was analyzed by real time qPCR. The expression level of *osteocalcin*, *osteopontin*, *osteonectin*, and *alkaline phosphatase* was 2-fold higher in the group with IGF2 supplementation. An *in vivo* experiment (critical-sized calvarial defect mouse model) showed the same results on the

regeneration of the damaged bone. In chondrogenic cell differentiation, the gene expression of chondrocyte-specific markers was analyzed by real-time qPCR. The expression level of *Decorin, Chordin-like 1, Pax1, Aggrecan, Collagen type II* and *MMP13* was higher in the group with IGF2 supplementation. The group supplemented with IGF2 showed a higher induction of chondrogenic differentiation. Both the osteogenic and chondrogenic induction data show that the supplementation of IGF2 promotes osteogenic and chondrogenic differentiation of PESCs. Collectively, this study presents the whole effective process from establishment to differentiation into osteogenic and chondrogenic cells of PESCs. The findings provide two insights on PESC research. First, as an alternative to the micro-drop culture, the MTC culture method is an effective and affordable embryo culture for generating PESC lines. Second, compensative supplementation of imprinting factor IGF2 may improve the efficiency of differentiation to specific lineages in monogenic stem cells.

Keywords: Embryo culture, Parthenogenesis, Parthenogenetic embryonic stem cells, Imprinting gene, Osteogenic cell, Chondrogenic cell

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REVIEW OF LITERATURE

Stem cells

Stem cells are undifferentiated cells that can differentiate into all functional cells (pluripotency) and can divide to produce other stem cells (self-renewal) [1]. In mammals, there are two types of stem cells: adult stem cells (ASCs) and embryonic stem cells (ESCs) [1]. In adult somatic tissues, ASCs function as progenitor cells, which act as a repair and regenerative system for the damaged adult tissues. ESCs, which are derived from the inner cell mass of a blastocyst, are pluripotent cells that can differentiate into all three germ layers (ectoderm, endoderm and mesoderm). Unlike ASC, ESCs have indefinite self-renewal capacity and pluripotency, so theoretically they can provide an unlimited source of cells for cell therapy [2]. These characteristics of ESCs were defined one of the most useful cell sources for tissue regenerative medicine.

Parthenogenesis and parthenogenetic embryonic stem cells

Parthenogenesis is a process of asexual reproduction in which the growth of embryos occur without fertilization [3]. In mammalians, parthenogenesis leads to growth and development of an embryo from an unfertilized oocyte.

Parthenogenesis occurs naturally in many plants, some invertebrate animal species and vertebrates such as fish [4]. In addition, parthenogenesis has been induced artificially in many species including mammals. Generally, mammalian parthenogenetic embryo cannot develop to full-term. So, these embryo technologies are widely used in research of pre-implantation embryo development and stem cell source. Parthenogenetic embryonic stem cells (PESCs) can contribute to the development of various tissues. Moreover, the use of PESCs can avoid ethical concerns surrounding human ESCs research because the parthenogenetic embryo itself cannot develop to full-term. These PESCs may improve organ transplantation efficiency by lessening the risk of major histocompatibility complex mismatch [5, 6]. Due to these reasons, many groups harvested and conducted research of PESCs from various animal species [5-9].

3D culture system of embryos

Numerous embryo technology research groups have developed and improved *in vitro* culture systems for the culture of fertilized or immature oocytes [10, 11]. These improvements have involved the chemical composition of the culture media. Indeed these approaches have proven extremely beneficial and have undoubtedly contributed largely to improved success rates following assisted reproduction [11-14]. However, not only do the chemical requirements of the developing embryo need to be considered, but physical

conditions and new culture devices may also be important factors in improved *in vitro* culture conditions [15-17]. Among these devices, micro well culture system is well researched. This device and method attempts to create a microenvironment in the individual or small groups of embryos and offers an advantage of increasing surface area point-of-contact between embryos. The most well-known micro-well approach is the well-of-the-well (WOW) system, as first described by Dr. Vajta [17, 18]. The WOW system has been used successfully with embryos from a variety of species including mouse, pig, cow, and human. It has also resulted in differing gene expression levels in bovine embryos compared to traditional micro-drop cultured [19-21].

Imprinting genes

Mammalian is inherited two copies of autosomal gene, from mother and father. Both copies are functional for the expression of specific genes. However, in several genes one copy is turned off or silenced in a parent-of-origin dependent manner. These genes are called imprinted or imprinting gene [22, 23]. One copy of the imprinting gene was epigenetically imprinted in the oocyte or the sperm. If the allele inherited from the father is imprinted, it is silenced, and the allele from the mother is occurred DNA transcription and gene expressed. Genomic imprinting has been demonstrated in variety of species such as fungi, plants and animals [24-26]. In the recent studies, there are about 150 imprinted genes known in the mouse and about 80 imprinted

genes in humans. Genomic imprinting is an inheritance process independent of the classical Mendelian inheritance. It is an epigenetic process that involves DNA methylation and histone modificationwithout altering the genomic sequence [27]. Imprinted genes are associated with numerous human pathologies because their abnormal haploid genomic state enables epigenomic errors to dysregulate imprinting gene function. Human diseases, Angelman syndrome and Prader–Willi syndrome, Alzheimer disease, autism, bipolar disorder and various cancers were reported to related genomic imprinting errors [28-30]. Nucleus transplantation experiments in mouse zygotes in the early 1980s confirmed that normal development requires the contribution of both the maternal and paternal genomes. The diploid mouse embryos (only one parental allele) derived from parthenogenesis die at the blastocyst or pre-implantation stage [31, 32]. Until recently, despite various researches, the detail mechanisms for genomic imprinting are still incompletely known. However, in mouse parthenogenetic embryo, methylation modification of the promoter of insulin like growth factor 2 (IGF2) has led to the expression of this gene and the birth of a live parthenogenetic mouse [33]. So, this thesis showed that exogenous supplement of protein derived imprinting gene promotes differentiation of murine parthenogenetic stem cells.

Embryonic stem cell differentiation into osteogenic lineage

ESCs have a self-renewal activity which gives rise to all somatic cell lineages [34]. Thus, ESCs were defined as one of the useful materials for tissue

engineering and regenerative medicine. Lineage specific differentiation of ESCs can be directed under specific induction conditions [35, 36]. Spontaneous differentiation of ESCs can be induced by the removal of the feeder cell layer or pluripotency factors in media such as leukemia inhibitory factor (LIF). This differentiation of ESCs formed 3D ESC aggregates known as 'embryoid bodies' (EBs) [37]. These cell groups exhibit regional specific differentiation into the derivatives of the three germ layers, the mesoderm, ectoderm and endoderm [37]. During *in vitro* EB formation, ESCs used a similar differentiation pathway as *in vivo* embryogenesis. After EB formation, EBs or dissociated cells from EBs are induction into an osteogenic lineage in a specialized induction media [38-40]. Previously research has shown that the addition of specific factors such as β-glycerophosphate, ascorbic acid, dexamethasone, retinoic acid and vitamin D3 resulted in the differentiation of ESCs into an osteogenic lineage. [39-41]. The differentiation statues were analyzed by specific osteogenic gene expression profiles or by the mineralization activity. [42]. Additionally, comparison studies have reported that the expression of osteogenic markers such as alkaline phosphatase and osteocalcin were highly enhanced in an ESC culture in a 3D scaffold culture system [43]. Taken all together, these findings show that ESCs can be used as a good source for bone tissue engineering.

Rationale

Current orthopedic and dental practitioners treat and cure bone damaged diseases including osteoporosis and age-related bone degenerative diseases. In many cases, surgery inserting metal or a biocompatible material is one of the therapeutic modalities currently used by dental practitioners. These treatments ameliorate the symptoms and return normal activity to the patient. However, these treatments cannot be used for several bone and cartilage diseases. Thus, studies on regenerative medicine are needed in the field of bone and cartilage therapy. ESCs are one of the most important cell sources as well as research subjects in regenerative medicine. Embryo culture and ESC line establishment have been constantly studied and improved over the past decade. However, the efficiency of the whole process is low. For ESCs to be a good material for regenerative medicine, increasing the efficiency of cell line establishment and differentiation is needed. Moreover, ethical concerns always arise using ESCs. Ethical concerns can be alleviated by using PESCs. Thus, if studies are done to overcome the limitations found in the differentiation of PESCs, these cells could be a good source material in regenerative medicine. Thus, the main goal of this thesis was to develop and confirm effective methods for the establishment of PESCs and their differentiation into osteoblast and chondroblast. To achieve this goal, I performed studies to evaluate the effect of the micro-tube embryo culture system on the generation of a parthenogenetic embryo stem cell line as well as the effect of a specific imprinting gene supplement, insulin like growth factor2 (IGF2), on the differentiation of PESCs into osteogenic and chondrogenic cells.

INTRODUCTION

ESCs are one of the most important cell sources and research subjects in regenerative medicine. ESC research in regenerative medicine includes from the establishment of stem cell lines to their differentiation into functional somatic cells. Procedures for embryo cultures and ESC line establishment have been constantly investigated and improved over the past decade. However, the efficiency of the whole process is low. Moreover, many of the mechanisms of cell differentiation are still unknown. For ESCs to be a good material for regenerative medicine, more research is needed on cell line establishment and differentiation efficiency into functional cells. Therefore, the aim of this study was to find a method to increase the efficiency of the whole process from cell line establishment to differentiation into functional cells using PESCs which is one of the ESCs.

1. Generation of parthenogenetic murine embryonic stem cells

This topic has been largely reproduced from an article published, entitled `Promoted expression of *Igf1, Dnmt3a* and *Oct4* in the parthenogenetic

murine blastocysts developed in an oil-free microtube culture system may support stem cell generation`[44].

Several properties of PESCs make them appealing as an alternative stem cell source. In addition, these cells are essential for understanding the differentiation process of mono-parental cells. Reportedly, when a parthenogenetic embryo is used to make ESCs and those cells are used to make chimeras, chimeric offspring having tissues from PESCs can be obtained [45]. This result suggests that the PESCs can contribute to the variable tissues of a developing embryo. Moreover, the use of PESCs can avoid ethical concerns surrounding human ESC research because the parthenogenetic embryo itself cannot develop to term. These PESCs may improve organ transplantation efficiency by lessening the risk of major histocompatibility complex mismatch [5, 6]. Due to these reasons, many groups harvested PESCs from various animal species, such as: mice [5, 6], monkeys [46, 47], rabbits [9], buffalos [8], and humans [7, 48]. In order to obtain PESCs, artificial oocyte activation and *in vitro* culture (IVC) processes are required. Many groups have developed IVC systems for culturing fertilized oocytes in mice, pigs, and cows by altering physical conditions and introduced culture devices [16, 17, 19]. From those reports, they were able to produce blastocysts efficiently. Typically, the starting material for generating mouse ESCs can be either a Day 3.5 expanded blastocyst or a further stage flushed from the uterine horns. In that system, the IVC process is not required to obtain the blastocyst. However, Liu et al. used IVC-derived blastocysts as

the starting material to obtain murine ESCs for the first time and showed that IVC systems for producing the blastocysts can influence the efficiency of ESC generation [49]. Since ESCs are derived from the inner cell masses (ICMs) of the blastocysts, the quality of the blastocyst can determine the rate of successful ESC establishment [49, 50]. For successful ESC generation, quality blastocysts should be provided efficiently. Previous reported that the micro-tube culture (MTC) system enhanced the developmental speed of embryos, blastocyst formation rate, and total cell number in blastocysts when compared with the conventional drop culture (Drop) system in the parthenogenetic murine embryo culture [51]. Because the embryos are placed together in the small area (<2 mm diameter) of the U-shaped microtube bottom, the MTC system provides embryos more opportunities to affect each other through potential paracrine actions than a conventional drop culture system.

In the present study, in order to increase the efficiency of mouse PESC isolation, I applied MTC culture system to obtain more quality parthenogenetic blastocysts. The parthenogenetic blastocysts cultured either by Drop or MTC were seeded onto feeder layers, and their ICM outgrowth and PESC line establishment rates were compared. In addition, the gene expression levels of *Igf1, Fgf2,* and *Egf* [for the cell growth] [52, 53] *Igf2* [for imprinting status] [54], *Dnmt3a* and *Dnmt3b* [for methylation levels] [55], and *Nanog* and *Oct4* [for the level of pluripotency] [56, 57] in the blastocysts

obtained by MTC and Drop were also analyzed by quantitative Real time-qPCR to compare their molecular potentials for generating PESC.

2. Osteogenic differentiation of parthenogenetic murine embryonic stem cells

This topic has been largely reproduced from an article published, entitled `Insulin-like growth factor 2 promotes osteogenic cell differentiation in the parthenogenetic murine embryonic stem cells'[58].

Hard tissue regeneration is an issue of critical importance in orthopedics and dental medicine. Many people that have bone diseases and damaged hard tissues required to exchange artificial prosthesis or bone tissue repairs. Major strategies to repair skeletal damage include the use of autogenous or allogenic bone grafts as well as various prosthetic implant devices [59]. However, these therapeutic methods have several shortcomings. The supply of suitable bone graft material is very limited and the biocompatibility of implant prostheses is a very serious issue after surgery. Therefore, regenerative skeletal repair using stem cells has become a topic of great interest in orthopedics and dentistry research. ESCs are permanent cells, which can be isolated from the inner cell mass of blastocysts in mice. These cells are pluripotent and are capable of self-renewal in specific culture environments. Upon withdrawal of leukemia inhibitory factor (LIF) or feeder cell supports, ESCs will differentiate into variable cell mass complexes called embryoid bodies (EBs). Differentiation

within these cell aggregates occurs in a defined temporal manner with the initial formation of all three germ layers, followed by further differentiation to terminally differentiated cell lineages [60, 61]. A variety of differentiated cell types have been generated from ESCs *in vitro* including neural cells, hematopoietic cells, cardiomyocytes, and osteoblasts [41, 62, 63]. The demand for regenerative approaches to treat bone defects is increasing, and ESCs may be an alternative option to bone grafting to achieve hard tissue regeneration. However, it is not easy to control ESC differentiation into specific cell types, and the use of ESCs is limited by the immune rejection response as well as the ethical debate surrounding embryo destruction. To avoid these problems, the use of ASCs, which have the same genetic information of the patient they were derived from, has been suggested. ASCs have been shown to facilitate bone repair in various osseous defect models [64, 65]. These cells have multipotent differentiation capacity and can address several problems related to tissue repairing. However, very few ASCs are present in the body and it is very difficult to isolate these cells. As result, it is very difficult to obtain sufficient quantities of these cells for therapeutic purposes [66]. In addition, the self-renewal capacity and the potential of specific cell type differentiation of ASCs decreased with the age increasing [66, 67].

In a previous study, I demonstrated that PESCs in mice can be induced to differentiate into osteogenic cells *in vitro*. PESCs are pluripotent, and if derived from an oocyte of a female patient, have that patient's genetic

information, thus preventing immune rejection responses in females. In humans, female are more susceptible to osteoporosis and other bone-related diseases than males [68]. Therefore, PESCs obtained from a patient's oocyte can potentially be used for organ or tissue regeneration therapy. PESCs have been shown to form three germ layers in a teratoma study [45]. This indicates that PESCs can contribute to the various tissues and organs of a developing embryo. Moreover, because the parthenote cannot develop to term, the use of PESCs avoids the ethical issues associated with human ESCs. PESC may also improve organ transplantation efficiency by reducing the risk of major histocompatibility complex mismatches [5, 6]. PESCs have been generated from various animals such as mice [5, 6], monkeys [46, 47, 69], rabbits [9], buffalos [8], and humans [7, 48, 70] for basic research. However, the use of PESCs in the field of regenerative medicine is only beginning to be explored. Although PESCs have many advantages compared to ESCs, these cells have some defects in their differentiation potential, especially differentiation into endodermal and mesodermal lineages [36].

Various proteins have been implicated in the formation of the three primary layers in the mouse embryo. These include growth factors and their receptors, cell adhesion and extracellular matrix molecules, and transcription factors. Studies in various mouse models have provided evidence that members of the fibroblast growth factor family, the Wnt family, the IGF family, and the transforming growth factor β super-family, such as activins and bone morphogenetic proteins, have mesoderm-inducing roles [36, 71, 72].

IGF2 is a very important factor for mesoderm formation in mouse embryonic development, and it may cause the biased determination of primitive ectoderm cells toward mesoderm cells or promote the selective proliferation of already determined mesoderm cells [72]. IGF2 is known to act both as a mitogen and a differentiation factor by triggering different signaling pathways at the same time [73, 74] Therefore, IGF2 may well cause both the determination and the proliferation of mesoderm cells. There are many reports that IGF2 and other member of the IGF gene family influence osteogenic cell activity and bone formation. Furthermore, it has been reported that IGF2 and IGF1 produced locally may modulate both osteoblast–osteoclast interactions and osteoblast formation, and thereby play an important role in bone remodeling [75, 76]. Bone homeostasis depends on the balanced action of bone resorption by osteoclasts and on bone formation by osteoblasts. Therefore, IGF2 may induce the osteogenic differentiation of stem cells.

The expression of imprinting genes related to growth and organ formation such as IGF2 is abnormal in parthenogenetic embryos, as these genes are only expressed by the paternal genome, which is absent in parthenotes. As an imprinting gene that is only expressed in paternal-oriented genomes, IGF2 have been shown to play an important role in regulating placental development and fetal growth [72, 77-79]. In the post-implantation embryonic period in mice, *Igf2* mRNA and protein are produced in the primitive endoderm at embryonic day 6.5 (E6.5), then in the extra-embryonic mesoderm cells they begin to appear (E7.0), and then, in the anterior-proximal

and lateral embryonic mesoderm cells (E7.5). Later, IGF2 becomes abundant in mesoderm derivatives such as the developing heart (E8.0) and somites (E8.5) [79]. Before E13.5, IGF2 signaling is transduced by the IGF1 receptor [80]. The IGF2 receptor is involved only in IGF2 degradation. Deletion of this gene leads to placental and fetal growth restriction, especially in the early stages of gestation [79, 81, 82]. Similarly, over-expression of IGF2 leads to placental and fetal overgrowth [77]. IGF2 enhances growth via paracrine and autocrine actions that stimulate cell proliferation and survival [72, 78]. IGF2 appears to be induced by placental lactogens and high concentrations of peptides and mRNA *in utero*, suggesting that IGF2 is important in fetal metabolism [83]. Thus, fetal and placental IGF2 appear to play an important role in regulating the relationship between fetal and placental growth and the placental capacity to transport nutrients, which occurs by facilitated and active transport [72]. In humans, contrasting to mice, IGF2 expression is maintained postnatally. The significance of this continued expression of IGF2 in humans is unknown [84].

Poly L-lactic acid (PLLA) scaffold was used for *in vivo* bone tissue regeneration experiment in this study. At present, PLLA is one of the most promising biopolymer. It has been the subject of an abundant literature including review articles [85-87]. As the degradable medical implant material, a large number of investigations have been carried out on the application of PLLA to the bone regeneration and drug delivery system in the shape of rod, plate, fiber and beads [88]. PLLA, a biocompatible polymer, is the most

extensively studied and used since it does not exert toxic or carcinogenic effect to the local tissues [89]. Moreover, PLLA can be degraded abiotically and the ultimate degraded product of PLLA is lactic acid, which is then incorporated into tricarboxylic acid cycle and excreted, showing non-toxicity. A variety of materials including biomimetic apatite and extracellular proteins such as fibronectin, collagen and vitronectin have been attached onto the surface of PLLA through either non-covalent or covalent attachment to control the interaction between PLLA and cells [89, 90]. Therefore, PLLA was used here as scaffold for *in vivo* experiment.

In the present study, I evaluated the effects of IGF2, which is important for organ formation in embryonic development, on *in vitro* osteogenic differentiation of PESCs and *in vivo* bone regeneration in a critical-sized calvaria mouse defect model.

3. Chondrogenic differentiation of parthenogenetic murine embryonic stem cells

This topic has been largely reproduced from an article published, entitled `Induced chondrogenic differentiation of parthenogenetic murine embryonic stem cells by insulin-like growth factor 2 treatment in a three-dimensional culture environment'[91].

Cartilage regeneration and repair are critical issues in orthopedics and dentistry. Cartilage is a structural body part that is part of the skeletal system

in animals, including humans. There are many people who suffer from diseases related to articular cartilage, such as osteoarthritis. In dentistry, degenerative joint disease occurring in the temporomandibular joint (TMJ) is one of the diseases rooted in the damage of articular cartilage. One notable feature of cartilage is that it is avascular. Thus, in cases of traumatic injury and age-related degenerative diseases associated with articular cartilage, normal mechanisms of tissue repair that involve the recruitment of stem or progenitor cells to the site of injury do not apply [92]. The unsatisfactory results of surgical and nonsurgical interventions for the repair of damaged articular cartilage are attributable to this poor healing capacity of cartilage [93].

Various strategies including the use of growth factors, tissue transplantation and cell transplantation therapy have been used in an attempt to reconstruct damaged articular cartilage [94]. Of these, cell transplantation therapy has proven to efficiently augment the numbers of chondrocytes in articular cartilage [95]. However, mature autologous chondrocytes have limited proliferative capacity, so they are unsuitable for providing adequate cell numbers for transplantation therapy. Moreover, the proliferative potential of chondrocytes decreases with patient age [96]. Thus stem cells have been used for cell transplantation therapy because of their self-renewal capacity. Bone marrow-derived mesenchymal stem cells (MSCs) have the potential to differentiate into various cells of mesenchymal lineages such as adipocytes, chondrocytes and osteoblasts [97]. However, the self-renewal and

proliferative capacity of MSCs are very much limited and decrease with age like mature chondrocytes [98]. In addition, during the *in vitro* expansion process, MSCs could lose their phenotype by spontaneous transformation, making them inadequate for damaged tissue repair [99].

ESCs, which are derived from the inner cell mass of a blastocyst, are pluripotent cells that can differentiate into all three germ layers. ESCs also have indefinite self-renewal capacity, so theoretically they can provide an unlimited source of cells for cell therapy [2]. However, it is difficult to control the differentiation process of ESCs into specific cell types, and there are still ethical concerns about the use of ESCs with regard to the destruction of developing embryos [100, 101]. An alternative approach is to use PESCs, which are derived from parthenogenesis. Parthenogenesis is a mechanism of reproduction in which the embryos are derived from oocytes without fertilization [102]. If PESCs are obtained from a female individual's own oocytes, the PESCs have that female individual's genetic information, so the immunological rejection response seen in the transplantation recipients who receive MSCs or ESCs from donors would not occur. Therefore, PESCs obtained from a female patient's oocyte can be potentially used for tissue regeneration. Moreover, because parthenogenetic embryos cannot develop into full-term embryos, the use of PESCs avoids the ethical issues associated with destruction of human embryos. PESCs have been researched in mice, non-human primates and humans [46, 48]. However, in the area of regenerative medicine, even though PESCs have many advantages compared

to ESCs, the utilization of PESCs has not been explored widely because they have some defects in differentiation potential, especially in differentiation into endodermal and mesodermal lineages [36].

In the present study, we chose to use transforming growth factor-beta (TGF-ß), bone morphogenetic protein-2 (BMP2) and bone morphogenetic protein-4 (BMP4) to induce the chondrogenic differentiation of murine PESCs. TGF-ß is thought to inhibit the differentiation of ESCs into mesenchymal cells with another growth factor, FGF2 [103]. However, these factors are also considered a promoter of the process by which mesenchymal cells differentiate into chondrocytes. BMP2 and BMP4 applied to EBs increased the development of chondrogenic cells constructing cartilage nodules [103]. BMP2 promotes chondrogenic differentiation in embryo development, which means BMP2 promotes the differentiation of ESCs into mesenchymal cells, and mesenchymal cell differentiation into chondrocytes is promoted by BMP4 [103, 104]. Moreover, BMP2 and BMP4, with other factors such as vitamin D_3 and dexamethasone, can induce the osteogenic and chondrogenic differentiation of mesenchymal cells. BMP2 induces the differentiation of ESCs into an osteoblast, chondrogenic or adipogenic fate depending on the supplementary cofactors provided [105].

The IGF family regulates the growth and development of many tissues by intracellular signaling pathways, especially in the prenatal period. The IGF signal system consists of two ligands (IGF1 and IGF2), and two kinds of cell surface receptors (IGF1R and IGF2R) [106, 107]. Both IGF1 and IGF2 bind

to IGF1R, and IGFBP-family also binds to the same receptor with individual specificities and affinities. On the other hand, only IGF2 can bind IGF2R. IGF2 is known to be active principally at early stages of skeletal development. However, the role of IGF2-associated with chondrogenesis is unknown, in comparison to IGF1, which is known to stimulate proteoglycan synthesis in chondrocytes via the phosphatidyl inositol 3-kinase (PI3K) pathway [108, 109]. Among IGF family members, IGF2 is known to be a significant factor for mesoderm formation in murine embryonic development and it may cause the biased determination of primitive ectodermal cells toward mesodermal cells or promote the selective proliferation of already determined mesodermal cells [36, 110]. IGF2 and the other members of the IGF family are known to influence osteogenic cell activity and bone formation [75]. Moreover, IGF2 is reported to induce the transcriptional activation of chondrogenic genes via intracellular signaling pathways when applied to human chondrocytes [111]. IGF2 is a product of an imprinted gene that is only expressed in the paternal genome, so the expression of this gene is abnormal in parthenogenetic embryos [79, 112].

In a previous study, we demonstrated that supplementation of osteo-induction medium with exogenous IGF2 significantly promotes osteogenic differentiation of PESC-derivatives both *in vitro* and *in vivo* [113]. We hypothesized that the lack of endogenous IGF2 in PESCs can be compensated for by exogenous supplementation of IGF2 [114]. As a result, the addition of soluble IGF2 could potentially enhance the osteogenic lineage differentiation

of PESCs. In this study, citing previous results, we present data demonstrating that PESCs can be differentiated into chondrogenic cells by induction medium containing multiple factors such as ascorbic acid, dexamethasone, BMP2, and TGF-β, and this can be promoted by the addition of exogenous IGF2, since the expression amount of IGF2 gene is insufficient in PESCs [115].

Taken together, in this thesis, I present data demonstrating that effective methods of PESCs generation and differentiation into osteogenic and chondrogenic cells. Using 3D embryo culture method, MTC and addition of soluble IGF2 in induction media could increase efficiency in the whole process on generation and differentiation of stem cell, and these results could provide useful information for regenerative medicine.

MATERIALS AND METHODS

Animals and chemicals

All inorganic and organic compounds were obtained from Sigma-Aldrich Korea (Yong-in, Korea) unless otherwise stated. Six-week-old C57BL6 X DBA2 F1-hybrid (B6D2F1) female mice were used as sources for the oocytes. All media for handling and culture of oocytes and parthenotes were based on CZB and KSOM [12, 116].

Recovery of oocytes, parthenogenetic activation, and in vitro culture (IVC)

All animal procedures were approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-061023-1). Five-to seven-week-old female C57BL6 x DBA2 F1 hybrid mice (B6D2F1) were superovulated with 5 IU equine chorionic gonadotropin followed by a second injection of 7.5 IU human chorionic gonadotropin (hCG) 48 h later. Oviducts were excised 15 h after hCG injection, and an average of 40 oocytes per mouse were obtained. Hyaluronidase (1 mg/ml) was used to remove cumulus cells, and oocytes were washed with Hepes-buffered CZB and exposed to an activation medium, consisting of 10 mM $SrCl_2$ with 5 μg/ml cytochalasin B in

calcium-free CZB for 5 h (Fig. 1). The activated oocytes were developed to the blastocyst stage in KSOM under two different culture conditions, which were described in previous work [51]. Briefly, the activated oocytes were cultured either in a micro-droplet on a 35-mm cell culture dish (BD Biosciences, San Jose, CA, USA) of oil covering (Drop) or in a 250 μl micro-tube (Axygen, Union City, CA, USA) at the same embryo density (MTC) (Fig. 2). The oocytes were equally distributed to either MTC or Drop in a volume of 10 μl KSOM and then incubated for 5 days at 37.5℃ under 5% CO_2 in air. After IVC, zona pellucida of the expanding blastocyst was removed by washing the embryo with acid-Tyrode solution.

Establishment and culture of ESC lines from parthenogenetic murine embryos

To generate PESCs, zona-free parthenogenetic blastocysts were transferred onto an STO feeder layer in gelatinized tissue culture plates (Nunc, Roskilde, Denmark) containing ESC medium consisting of Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids (NEAA), 0.1 mM β-mercaptoethanol and 1,000 U/ml ESGRO LIF (Chemicon, Temecula, CA, USA). After 5 days of culture, the outgrown clumps derived from the inner cell mass (ICM) were counted under a stereo-microscope. The cell clumps were further trypsinized with 0.05% trypsin-

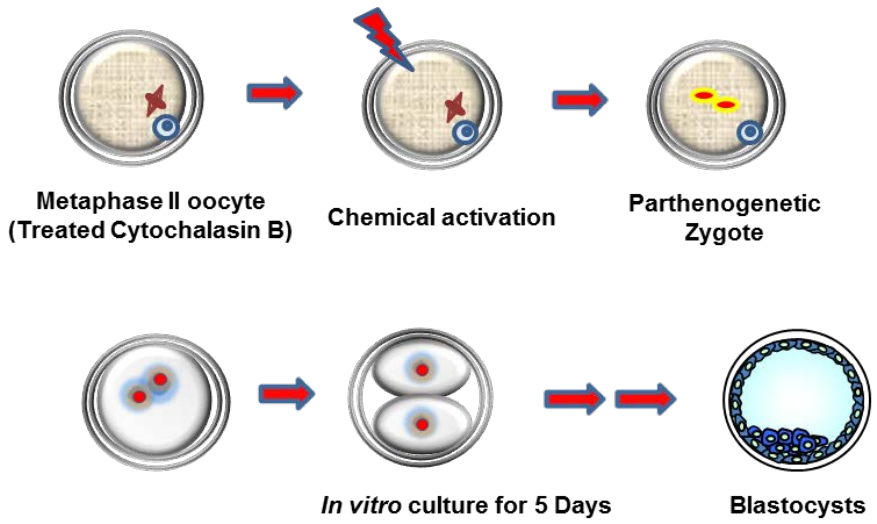


Figure 1. Diagram for methods of artificial murine parthenogenetic embryo generation.

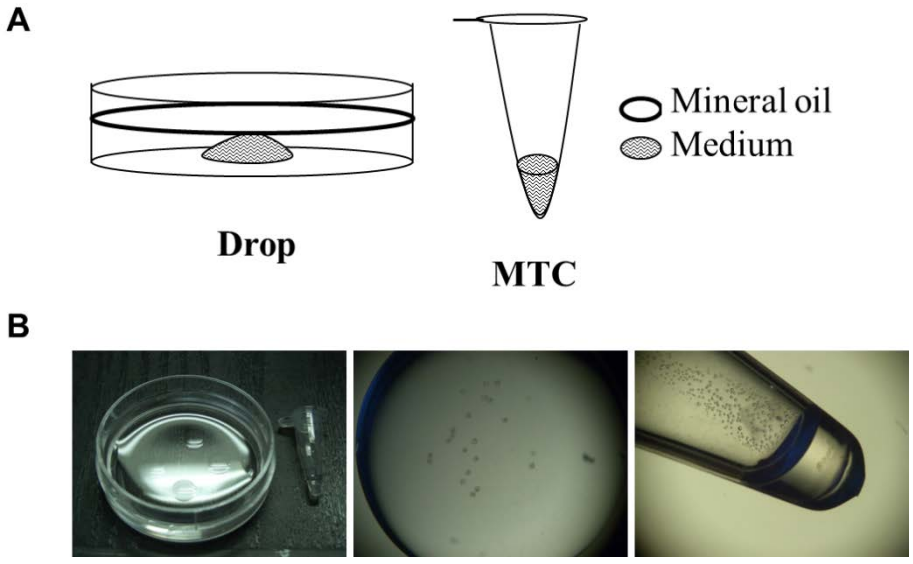


Figure 2. MTC embryo culture system. (A) Diagram of traditional Drop culture and MTC. (B) Embryos were cultured to the blastocyst stage in 5 μl volume of KSOM under MTC system and in 10 μl drop. Embryos were observed under the inverted microscope. Scale bar= 1 mm.

EDTA and seeded onto new gelatinized 4-well dishes with fresh ESC medium to generate PESCs. When the colonies appeared, they were considered to be at passage 0 and were propagated gradually under stringent culture conditions with careful monitoring and medium changes to ensure that the cells remained undifferentiated. The PESCs were cultivated on a feeder layer of mitomycin C-treated STO cells in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 50 U/ml penicillin, 50 mg/ml streptomycin, and LIF (1,000 U/ml) in an atmosphere of 37°C, 5% CO_2 in air, and the medium was changed once every day. ESCs from fertilized embryos of the same murine strain were used as a control. Both ESCs and PESCs after 30 or more passages were used for the experiments.

Characterization of mouse PESC lines

The PESCs after 20 or more passages were characterized by their expression of pluripotency markers. The PESCs that were grown on the cover slip coated with 0.1% gelatin were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. Fixed cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 min and washed three times. After blocking with 1% bovine serum albumin in PBS for 30 min, cells were incubated with the first antibody. Antibodies used in this experiments were: mouse monoclonal OCT4 (Santa Cruz, CA, USA), mouse monoclonal stage-specific embryonic antigen (SSEA)-1 and SSEA-4. After washes with PBS, primary antibodies

were applied using FITC-conjugated goat anti-mouse (Jackson Immunoresearch, West Grove, USA). Alkaline phosphatase (ALP) staining was processed by manufacturer's instruction (Chemicon, CA, USA). The PESCs were fixed with 4% paraformaldehyde for 2 min and rinsed for 5 min with rinse buffer (20 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, 0.05% Tween-20). During the rinse, reagents for ALP staining were prepared, as follows: Fast Red Violet with Naphthol AS-BI phosphate solution and water in a 2:1:1 ratio. After the stain solution was added, the cells were incubated in the dark for 15 min and washed with PBS, then observed.

In vivo differentiation (teratoma assay)

Early passage PESCs ($2x10^6$ cells per mouse) were injected subcutaneously into the rear thigh of a nude mouse (Balb/c, male). The ESCs from fertilized embryos (J1, 129Sv origin) were used as control. After 4 to 6 weeks, the mice with teratomas of 1-2 cm diameter were killed, and then the teratoma tissues were excised, fixed in 10% buffered formalin phosphate (Fisher, Loughborough, UK), embedded in paraffin, and sectioned for histological analysis.

In vitro differentiation (embryoid bodies analysis)

PESCs were differentiated *in vitro* in a suspension culture without LIF. Floating ESC aggregates, called embryoid bodies (EBs), were formed in a

bacteriological dish. On the second day of culture, EBs were transferred to a 15 ml tube and allowed to stand for 5 min until the EBs settled to the bottom of the tube. Then, the EBs were transferred to a new bacteriological dish and cultured for another 2 days. After 4 days in suspension culture, the gene expression levels representing 3 germ layers, AFP (endoderm), BRACHURY-T (mesoderm), and NESTIN (ectoderm), were measured by RT-PCR and real-time qPCR.

Formation of EBs (stem cell differentiation)

To induce EB formation, ESCs or PESCs on the culture plates were trypsinized for 2 min at 37°C. Trypsinization was terminated by addition of DMEM containing 10% FBS. After a brief centrifugation, cells were resuspended in medium followed by medium supplementation with 10% FBS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 50 U/ml penicillin, 50 mg/ml streptomycin, 10^{-7} M retinoic acid, and were then treated with or without different concentration of IGF2 (50 ng/ml on osteoblast differentiation and 1-100 ng/ml on chondrocyte differentiation). Cells were plated onto bacteriological-grade petri-dishes in the absence of fibroblast feeder layers and LIF. ESCs were maintained in culture for 7 days and the medium was replaced every 2 days. After 7 days of culture, ESCs formed free-floating aggregates or EBs (Fig. 3).

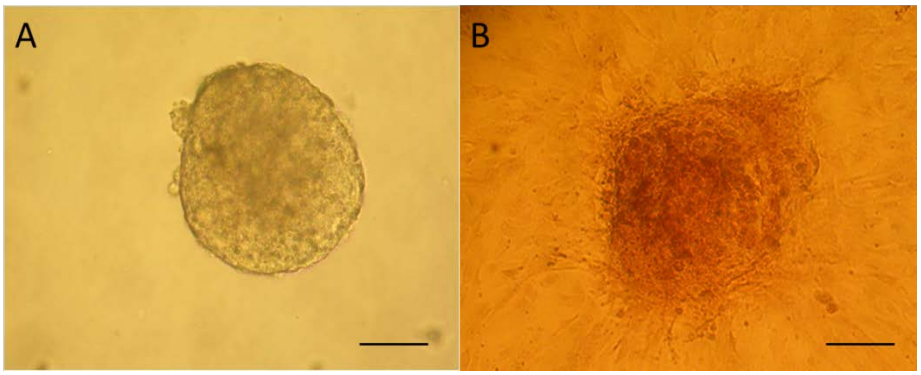


Figure 3. Photos of an embryoid body (EB). (A) A well-formed EB from PESCs shaped like an ellipse. (B) After 3 days of culture in osteogenic induction medium, EBs attached and expanded on the bottom of the plate. Bar length, 1 mm.

In vitro osteogenic induction of EBs

To induce osteogenic differentiation of EBs, we used a modified version of the osteogenic induction protocol of Buttery et al. (2001). Briefly, EBs were resuspended in α-modified Eagle's medium (α-MEM) containing 10% FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin, and allowed to adhere to 6-well culture plates at a density of three EBs/well. The medium was then additionally supplemented with 50 mg/ml ascorbic acid, 10 mM *β*-glycerophosphate, with or without 50 ng/ml IGF2. The EBs were maintained in culture for 20 days and the medium was replaced every 3 days.

Preparation of scaffolds and cells for calvarial implantation

The PLLA scaffolds were prepared as described in a previous report with some modifications [117]. In brief, the PLLA was dissolved in a mixed solvent (dioxane:dimethylcarbonate; 8:2) to make a 5% (w/v) solution. A solid–liquid phase separation technique and a subsequent solvent sublimation process were used to generate the porous PLLA scaffolds. The PLLA/dioxane solution was cooled to -20^{o}C for 2 h and transferred to -80^{o}C for additional 24 h. The frozen mixtures were freeze-dried at -10 ~ -5^{o}C of ice/salt bath for 7 days, and then stored in a desiccator. The PLLA disks with a diameter of 4 mm and thickness of 1 mm were prepared. The scaffolds were sterilized with ethylene oxide gas. After sterilization, scaffold samples were soaked at 37°C in general growth medium (DMEM, 10% FBS, 1% penicillin/streptomycin)

for 24 h. Then, scaffold samples were soaked in serum that had been extracted from B6D2F1 mice. After soaking, scaffold samples were washed with PBS. In preparation for implantation, IGF2-naive PESC and IGF2-treated PESC derivatives were seeded onto the scaffold. Following trypsinization, ten thousand cells were re-suspended in one sample volume of general growth medium (8.0×10^4 cells/cm^2), and seeded directly onto the scaffold. The same amount of medium without cells was used as an empty scaffold control. Before implantation, cell-seeded scaffolds were submerged at 37°C in medium and incubated for 24 h. One day after seeding, the samples were embedded in paraffin and cut in 5 μm sections and then routine hematoxylin and eosin (H&E) staining was performed to ensure the state of seeded cells.

Scanning electron microscopy (SEM)

One day after seeding, samples were washed twice with PBS, and fixed using 2.5% glutaraldehyde and 2% paraformaldehyde in pH 7.2 PBS for 24 h. After fixing, the samples were washed twice with PBS and post-fixed using 1% osmium tetroxide for 1 h and followed by washing twice with distilled water. Then the specimen was dehydrated by dipping it in increasing concentrations of ethanol and then by critical point drying. After drying for 24 h, the specimens were sputter-coated with gold epaladium and observed under an SEM at 15 kV (FE-SEM Hitachi S-4700, Tokyo**,** Japan).

Surgical procedures

Mice of B6D2F1 strain (25–30 g) were used as study subjects. All animal experiments including animal management and surgical procedures were approved and performed under the guidelines of the Institutional Animal Care and Use Committee of Seoul National University (approval number: SNU-061023-1). The animals were anesthetized with a subcutaneous injection of a mixture of Zoletil^{TM} and xylazine (30 mg and 10 mg per kg, respectively). The scalp covering the calvarial vault was shaved and scrubbed with betadine solution. An incision was made along the midline. Full thickness skin and the periosteum were raised to expose the calvarial bone surface. Careful drilling with a 4-mm diameter trephine bur was performed around the sagittal suture, and a standardized, round, segmental defect was made (Fig. 4). During drilling, the area was irrigated with saline solution and the underlying dura mater was maintained intact. A PLLA scaffold, with or without cells, was placed in the calvarial bone defect. The periosteum and skin were closed in layers with absorbable 5-0 chromic catgut (WRHI, Namyangju, Korea) non absorbable 4–0 black silk (Ethicon, Edinburgh, UK) sutures, respectively. Mice were sacrificed 10 weeks after the implantation. Calvarial bone was excised with careful trimming. The specimen was fixed in 10% neutral buffered formalin solution at 4°C for more than 12 h.

Figure 4. Surgery of critical-sized calvarial defects in mice

Micro-CT

Soft X-ray of the excised calvarial specimens was taken with a condition of 30 kV, 1.5 mA, 40 sec of exposure, and 25 cm of distance. Then, the calvarial bone specimens were examined using a micro-CT machine (Skyscan 1072; Skyscan, Aartselaar, Belgium). Specimens were placed on a cylindrical sample holder with the coronal aspect of calvarial bone in a horizontal position to ensure parallel scanning conditions. The pixel size was 17.99 μm. Image files were reconstructed using a modified Feldkamp algorithm, which was created using microtomographic analysis software (TomoNT; Skyscan). After the 3D visualizing process, bone volumes were measured in the region of interest. In addition, micro-CT scan was also performed in animals of which defects were not treated with any cell, serving as the blank control. The data were presented as average and standard error of means. The one-way ANOVA test was performed to compare the differences among the experimental groups. Number of samples in each group was four.

Histologic evaluation

Specimens were decalcified in 10% EDTA in 0.2 M $NaPO_4$, pH 7.4, for 7 to 10 days. The decalcified specimens were embedded in paraffin and cut in 5-μm sections, and then H&E staining was performed for histomorphological analysis to evaluate hard tissue formation in the bone defects.

Chondrogenic induction of EBs in two-dimensional (2D) or three-dimensional (3D) culture systems

In the 2D culture system, the EBs were re-suspended in alpha-modified Eagle's medium (α-MEM) containing 10% FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin and allowed to adhere to six-well culture plates at a density of five EBs per well. In chondrogenic induction medium (CIM), 10 mM dexamethasone, 10 μg/ml ascorbic acid, 10 ng/ml TGF-*β*, 5 ng/ml BMP-2 and 5 ng/ml BMP4 were supplemented and the EBs were maintained in culture for 21 days. The medium was replaced every two days. Alginate beads were used in the 3D culture system. Thus, preparation of alginate beads was the first step in the 3D culture system. Low viscosity alginate (1.2%, w/v) was dissolved in 0.15 M NaCl. Prior to use, the preparation was autoclaved for 20 min. EBs were suspended at the desired concentration in the alginate solution (50 EBs/ml), thoroughly mixed with gentle pipetting, and transferred to a 5-ml syringe equipped with a 22-gauge needle. The EBs and alginate mixture were slowly expelled in a drop-wise fashion into 10 ml of gently agitated 102 mM CaCl solution. Gelation occurred instantaneously. The alginate beads were cured at room temperature for 10 min. The CaC1 solution was decanted and the beads were washed sequentially four times in 0.15 M NaCl and once in CIM (Fig. 5). The encapsulated cells were cultured in six-well culture dish maintained at 37℃ under 5% CO_2. Each flask contained approximately 20-30 beads bathed in 7 ml of CIM and different concentrations of IGF2 in each

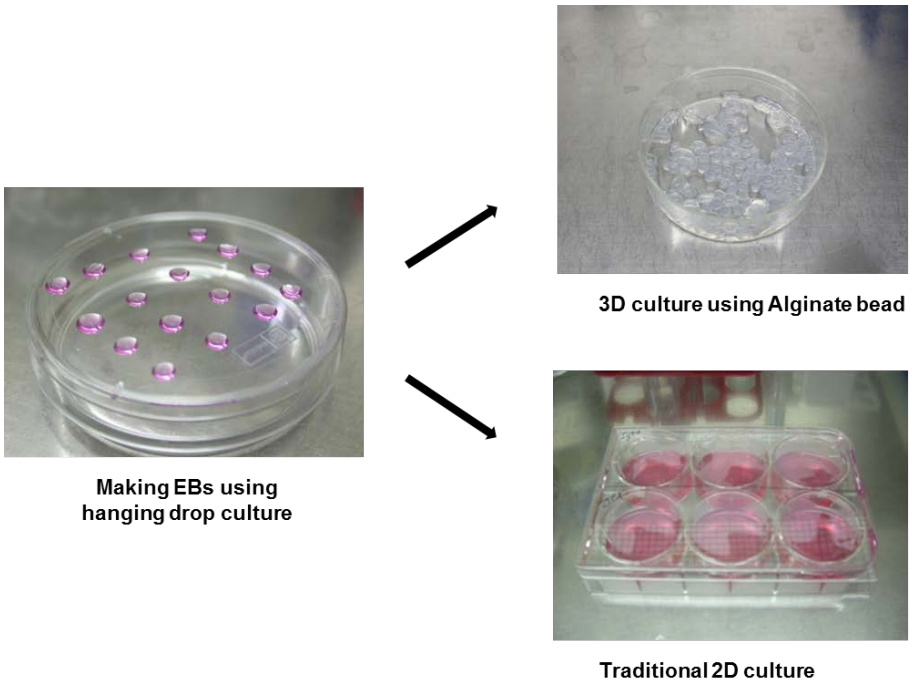


Figure 5. Diagram of two type on stem cells differentiation. EBs were made on hanging drop culture methods. After making EBs, EDs were differentiated into osseous tissues on 2D or 3D culture methods

experimental group (0, 1, 10, and 100 ng/ml). The medium was replaced every two days.

Alginate bead solubilization and cell recovery

In order to induce solubilization, alginate beads were incubated with a solution containing 10 mM MOPS, 100 mM sodium citrate and 27 mM NaCl for 10 min at 37℃. After incubation, the alginate beads immediately disintegrated, and the cells were recovered by centrifugation. For staining, the recovered cells were re-seeded and cultured in a six-well plate for 24 h.

Isolation of chondrocytes from the mouse

The murine chondrocytes isolation was performed as described in the published standard protocol by other research group [118]. Briefly, chondrocytes were prepared from the knees of five- to six-week-old B6D2F1 mice. The mice were placed in the face-down position on the experimental bench, and the skin and tissues were removed from their hind legs. The femurs were dislocated, and the joints tissues were discarded. Isolated femoral heads and femoral condyles were incubated in collagenase D solution (3 mg/ml collagenase D in DMEM supplemented with 2 mM L-glutamine, 50 IU/ml penicillin) for 60 min and then rinsed twice with PBS. The tissue fragments were agitated until all soft tissues detached from the cartilage pieces, and the fragments were then incubated with collagenase D solution

(0.5 mg/ml collagenase D) overnight. This method yielded a cell suspension, which was mixed thoroughly to disperse any cell aggregates, thus producing a suspension of isolated cells, which was centrifuged for 10 min at 450 X *g*. The pellet of chondrocytes was washed with PBS and re-suspended in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 50 IU/ml penicillin.

Cell staining

For Alcian blue and Safranin O staining, the culture plates were rinsed twice in PBS and then the cell culture plates were fixed with 4% formaldehyde in PBS for 30 min, washed once with PBS, rinsed with distilled water (dH_2O), and then processed by Alcian blue staining. Specimens were incubated with 1% (w/v) Alcian blue solution for 20 min or 0.1% Safranin O solution for 5 min. Excess stain was removed by washing in PBS, rinsing in 5% acetic acid to remove nonspecific staining, and then washing in PBS again. To compare 2D culture and 3D culture in similar environment, recovered 3D cultured cells were re-seeded and cultured in a six-well plate for 24 h like 2D culture.

mRNA extraction and cDNA synthesis

RNA was extracted from all samples using TRI reagent (Invitrogen). The all samples of each experiment were harvested, resuspended in 1ml of Trizol reagent by vortexing and then incubated for 15 min in ice. Then 0.2 ml of

chloroform was added, mixed by smooth shaking and incubated for 15 min. After centrifugation at 13,000*g* for 15 min at 4°C, the colorless upper aqueous phase was transferred to new tubes containing 0.5 ml of isopropanol and by smooth shaking and incubated for 15 min. Total RNA pellet was obtained by centrifugation at 13,000*g* for 10 min at 4°C, air-dried, and re-suspended in diethyl pyrocarbonate-treated water. The isolated RNA samples were used for Real time PCR analysis. For the synthesis of cDNAs, reverse transcription was performed for 1 h at 42°C in a final reaction volume of 25 μl containing the total RNA, 5 μl of 5 X reaction buffer, 5 μl of dNTP, 2.5 μl of synthesis primer, 0.5 μl of RNasin Plus RNase Inhibitor and 1 μl of M-MuLV reverse transcriptase (Promega, WI, USA). cDNAs were diluted by the addition of 50 μl of RNasefree ultra-purified water.

RT-PCR

At an early passage, trypsin-digested PESC and ESC cells were incubated in the bacteriological dish for 20 min to allow feeder cells to attach to the plates; then, PESCs and ESCs were harvested carefully. This step was repeated three times to remove feeder cells from the ESCs. RNA was extracted from undifferentiated PESCs/ESCs and EB using TRI reagent (Invitrogen). Reverse transcription for complementary DNA synthesis was performed with 6 μg RNA per sample using MMLV reverse transcriptase (Promega, WI, USA). Primer sequences were described in Table 1.

Real-time qPCR

Expression levels of developmentally important genes *(Igf1, Fgf2, Egf, Igf1, Dnmt3a, Dnmt3b, Nanog and Oct4)*, three germ layer marker genes *(Afp, Nestin, Brachury-t)*, osteogenic cell-specific genes *(Osteocalcin, Osteonectin, Bone sialoprotein, Osteopontin, Collagen type I, Alkaline phosphatase, and Runx2)* and chondrogenic cell-specific genes, *(Chordin-like 1, Collagen type II, Aggrecan, Decorin, MMP13 and Pax1*) were measured by real-time qPCR. Real-time qPCR primers were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). Real time PCR was performed using the ABI PRISM 7500 system and SYBR Green PCR Master Mix (Applied Biosystems). All samples were run in triplicate to obtain technical replicates. The primers list of target genes was showed in Table 1. In each run, 1 μl cDNA was used as a template added to 5 μl double-distilled water, 2 μl forward and reverse primers (20 pmol/ml), and 10 μl SYBR Green PCR Master Mix. The following amplification procedures were employed: denaturation stage (95°C for 10 min), amplification and quantification stage repeated 40 times (94°C for 15 sec, 60°C for 1 min with single fluorescence measurement) and dissociation curve stage (temperature increments of 0.1°C per 30 sec from 60 to 95°C with fluorescence measurement). Gene expression was always related to expression of Murine *Gapdh* as housekeeping gene, which is known to be a good reference gene for normalization of target genes expression levels. Quantification was performed using the $^{\Delta\Delta}$CT method. Non-template control was used as the negative control. The paired samples *t*-test

was performed to compare the differences among the experimental groups. Primer sequences were described in Table 1.

Statistical analysis

Outgrowth rates were analyzed using the Chi-square test of SPSS (SPSS Inc., IL, USA). Mean gene expression values were analyzed using the *t*-test to compare parameters between the different groups. Difference at $p < 0.05$ was considered significant.

Table 1. Primer sequences (5'-3') used in real-time PCR.

Gene name	Forward primer	Reversed primer	Access numbers
Oct4	CCGTGTGAGGTG GAGTCTGGAG	GCGATGTGAGT GATCTGCTGTAG	NM_013633.1
Nanog	GAAATCCCTTCCC TCGCCATC	CTCAGTAGCAG ACCCTTGTAAGC	NM_028016.3
Alpha-fetoprotein	CACACCCGCTTCC CTCATCC	TTCTTCTCCGTC ACGCACTGG	NM_007423.4
Brachury-T	CACACCACTGAC GCACAC	GAGGCTATGAG GAGGCTTTG	NM_009309.2
Nestin	GGAGAAGCAGGG TCTACAG	AGCCACTTCCAG ACTAAGG	NM_016701.3
Dnmt3a	GATGTTCTTTGCC AATAACC	CAGGAGCCCTGT AGAATC	NM_007872.4
Dnmt3b	CCTGCCCGCAAA GGTTTAT	GGCCACAACATT CTCGAACA	NM_010068.5
Fgf2	ATGGCGTCCGCG AGAAG	AGGTACCGGTTG GCACACA	NM_008006.2
Egf	GGTCCTGCTGCTC GTCTTG	TCCGCTTGGCTC ATCACAA	NM_010113.4
Igf1	GACAGGCATTGT GGATGAGT	GATAGAGCGGG CTGCTTTTG	NM_010512.5
Igf2	CCCAGGTGTTTGC CTCAACT	ATTAGGTTTGCG AGCGTTAA	NM_010514.3
Gapdh	GCATGGCCTTCCG TGTTCCTA	CTTCAGTGGGCC CTCAGATGC	NM_008084.3
Osteocalcin	CCGGGAGCAGTG TGAGCTTA	TAGATGCGTTTG TAGGCGGTC3'	NM_001032298.3
Osteonectin	ATCCAGAGCTGTG GCACACA	GGAAAGAAACG CCCGAAGA	NM_009242.5
Bone sialoprotein	CAGAGGAGGCAA GCGTCACT	CTGTCTGGGTGC CAACACTG	NM_009263.3

Osteopontin	GATGCCACAGATG AGGACCT	CTGGGCAACAG GGATGACAT	NM_001204201.1
Collagen type I	GCATGGCCAAGA AGACATCC	CCTCGGGTTTCC ACGTCTC	NM_007742.4
Alkaline phosphatase	GTGCCCTGACTGA GGCTGTC	GGATCATCGTGT CCTGCTCAC	NM_007431.3
Runx2	GTGCGGTGCAAA CTTTCTCC	AATGACTCGGTT GGTCTCGG	NM_009820.5
Pax1	GATGGAAGACTG GGCGGGTGTG	TTCTCGGTGTTT GAAGGTCATTGC	NM_008780.2
Chordin-like 1	TGCGAATACAAT GGAACCACTTA	ACAATGCCAAA TGCTCGTAGAT	NM_031258.3
Collagen type II	CTGCTCATCGCCG CGGTCCTA	AGGGGTACCAG GTTCTCCATC	NM_031163.3
Aggrecan	CCAAGTTCCAGG GTC ACTGTT	TCCTCTCCGGTG GCAAAGAAG	NM_007424.2
Decorin	CCCAGATCAGAA CAC TGCACC	ATGACCCTGACA ATCCCCTG	NM_007833.6
MMP13	CAGTTGACAGGCT CCGAGAA	CGTGTGCCAGA AGACCAGAA	NM_008607.2

RESULTS

1. Generation of parthenogenetic murine embryonic stem cells

ICM outgrowth rate of blastocysts derived either from MTC or drop methods

The blastocyst outgrowth rate was different significantly between the MTC (75.0%) and the Drop culture group (65.4%) (Table. 2). Although outgrowing inner cell masses were found in both groups after seeding on the feeder layers, three PESC lines had been only generated from the MTC system while no PESC line was generated from the blastocysts cultured via the Drop method.

Characterization of PESCs

The PESCs derived via MTC showed typical mouse ESC morphology (stacked like a dome or an oval with clear boundaries), and the cells adhered tightly to each other, making it impossible to visualize individual cells in the colonies. Immuno-staining showed that the PESCs lines are positive for pluripotent mouse stem cell markers, including OCT4, SSEA-1, and ALP (Fig. 6). In addition, RT-PCR analysis revealed that pluripotency genes *Oct4* and

Nanog are expressed in PESCs. The figures presented here (Fig. 6 to 8) were from the first PESC cell line derived from the B6D2F1 blastocyst (BDF-PESC1).

In vitro differentiation: EB

The differentiating potential of EB was analyzed using RT-PCR. After 4 days in suspension culture, only PESC-derived EBs expressed genes representing mesoderm (*Brachyury-T*) and ectoderm (*Nestin*), not endoderm (*Alpha-fetoprotein*) (Fig. 7) while ESC-derived EBs expressed genes representing three germ layers.

In vivo differentiation

Teratomas derived from PESCs showed only ectodermal and mesodermal lineage differentiation (Fig. 8). Rosettes of neural epithelium (Fig. 8A), gut-like epithelium (Fig. 8B), stratified squamous cells (Fig. 8C), and fibrous tissues (Fig. 8D) were shown in ESC-derived teratomas, while immature neural tissues (Fig. 8E), gut-like epithelium (Fig. 8E), neural tube structure (Fig. 8F), blastemal tissue (Fig. 8G), and glandular cells (Fig. 8H) were shown in PESC-derived teratomas. No endodermal tissue was observed in PESC-derived teratomas.

Table 2. The outgrowth and PESC generation from the blastocysts of B6D2F1 mice

Group[1]	Blastocysts	Outgrowth on the feeder layer (%)	PESC line
Drop	26	17 (65.4%)	0
MTC	32	24 (75.0%)	2

Three replicates.

[1]Drop: conventional micro-drop culture, MTC: microtube culture.

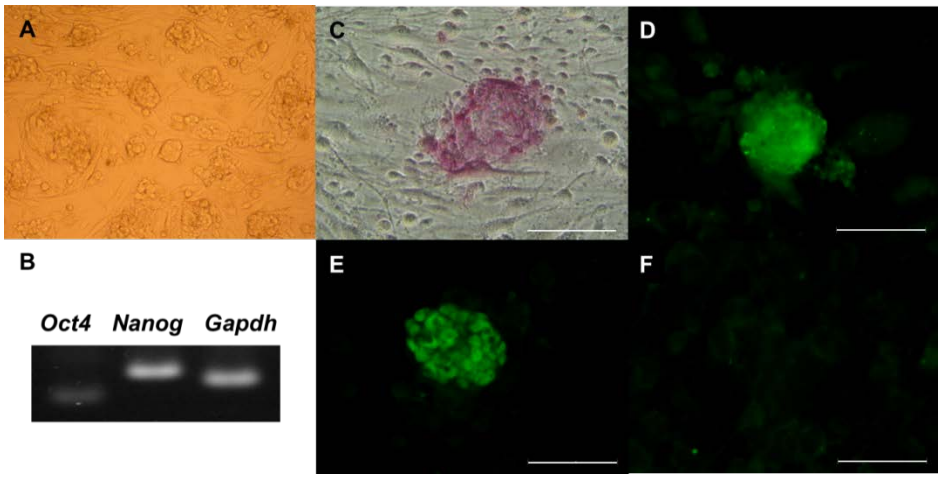


Figure 6. Parthenogenetic embryonic stem cells (PESCs) derived from the mouse parthenogenetic blastocysts (C57BL/6 x DBA2 F1-hybrid: B6D2F1) produced in a micro-tube culture system (MTC). The figure is from the first PESC cell line derived from the B6D2F1 blastocyst (BDF-PESC1). (A) Outgrown cell colonies at passage 4. (B) The PESCs showed the expression of the pluripotency marker genes, *Oct4* and *Nanog*, by RT-PCR. (C-F) The expression of pluripotent stem cell-specific markers was confirmed by immunostaining. The PESCs were positive for alkaline phosphatase (C), stage specific embryonic antigen-1 (D), and OCT4 (E) while negative for stage-specific embryonic antigen-4 (F). Scale bar= 100 μm.

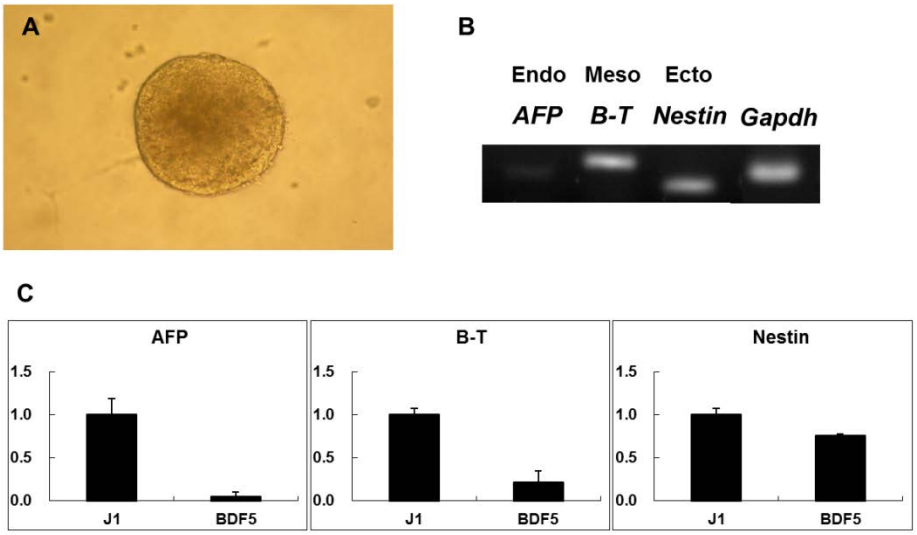
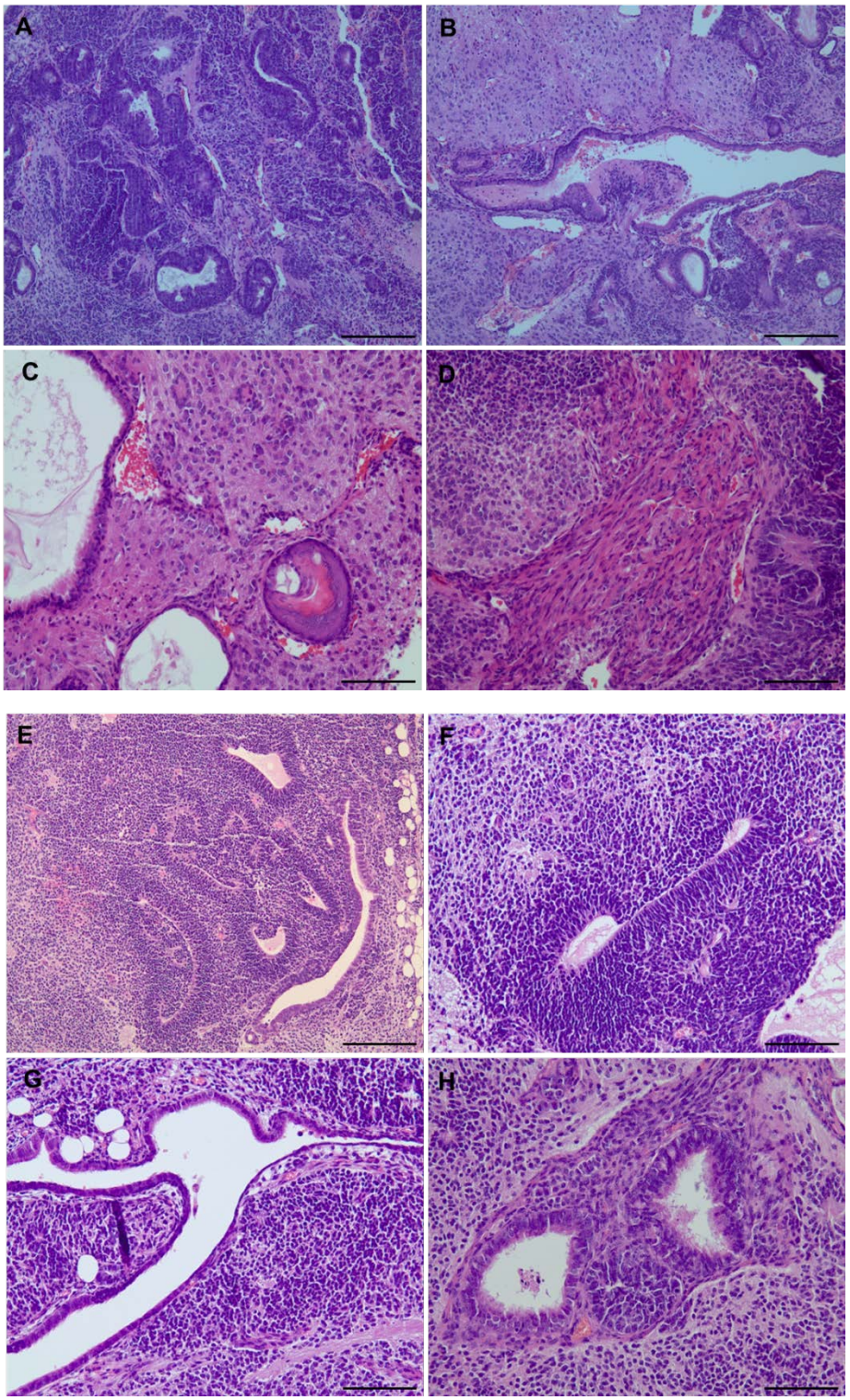


Figure 7. Differentiating potential of the embryoid body (EB) derived from the PESC. (A) Spherical EB was formed from the PESC (BDF-PESC1). (B) Expression of endodermal (*Alpha-fetoprotein, AFP*), mesodermal (*Brachyury-T, B-T*), and ectodermal (*Nestin*) markers in EB were analyzed by RT-PCR. The EB from PESC does not show endodermal differentiation potential. (C) The graphs show gene expression level of differentiating EB derived from PESC and ESC measured by real time RT-qPCR. Expression of endoderm-specific marker gene, *AFP* is not detected in PESC.


A
B
C
D
E
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G
H

Figure 8. Transplantation of ESCs or PESCs from B6D2F1 embryos under the skin of the nude mouse. (A-D) Histological analysis of the ESC-derived teratoma. Rosettes of neural epithelium (A), gut-like epithelium (B), stratified squamous cells (C), and fibrous tissues (D) are shown in ESC-derived teratomas. (E-H) Histological analysis of the PESC-derived teratomas. Immature neural tissue (middle and left) and gut-like epithelium (right) (E), neural tube structure and blastemal tissue (F), gut-like epithelium (G), and glandular cells (H) are shown in PESC-derived teratomas. Scale bar= 200 μm for (A, B, E) and 100 μm for (C, D, F, G, H).

Gene expression analysis in parthenogenetic murine blastocysts

Gene expression levels of *Igf1*, *Dnmt3a*, and *Oct4* were significantly ($p < 0.01$) higher in MTC-derived blastocysts than Drop culture-derived blastocysts (Fig. 9). *Igf1* and *Oct4* gene expression in MTC-derived blastocysts were 4-fold higher than in Drop culture-derived blastocysts. Gene expression levels of other genes, such as *Fgf2, Igf2, Dnmt3b, Nanog and Egf*, were not significantly different between the two groups.

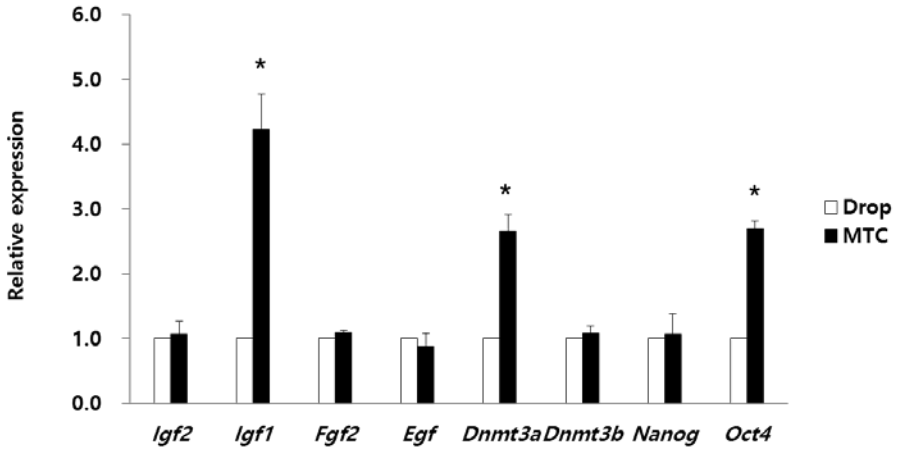

Figure 9. The expression levels of developmentally important genes on Drop and MTC cultured blastocyst. The graph represents the expression levels of developmentally important genes, such as *Igf1, Igf2, Fgf2, Egf, Dnmt3a, Dnmt3b, Nanog* and *Oct4*, in parthenogenetic B6D2F1 blastocysts derived from either MTC or Drop, as measured by real time RT-qPCR. Gene expression levels of *Igf1*, *Dnmt3a,* and *Oct4* are significantly higher (*$p < 0.01$) in MTC-derived blastocysts than Drop culture-derived ones.

2. Osteogenic differentiation of parthenogenetic murine embryonic stem cells

Bone related gene expression in differentiated cells

Expression levels of all the genes analyzed were significantly higher in IGF2-treated PESC derivatives than IGF2-naive PESC-derivatives (Fig. 10). Expression levels of *Osteopontin* (p = 0.017), *Osteonectin* (p = 0.021), *Osteocalcin* (p = 0.042) and *Runx2* (p = 0.032) were also significantly higher in IGF2-treated PESC derivatives than ESC derivatives whereas the levels of the other genes were similar to those in ESC derivatives.

Bone regeneration potential in murine calvarial defects

The internal structure of scaffolds was evaluated by SEM. The morphologies of cultured cells in the PLLA scaffolds were evaluated by SEM and histological staining on day 1. As shown in Fig 11A, the prepared PLLA scaffolds were highly porous. The irregular shaped pores were interconnected, and the size of individual pores ranged from several tens to two hundreds micron (67 ± 22 μm, n=176). One day after seeding, seeded cells in the scaffold showed well attached status on scaffold pores (Fig 11B, C). H&E staining confirmed that live status of cells at the time of staining (Fig 11D).

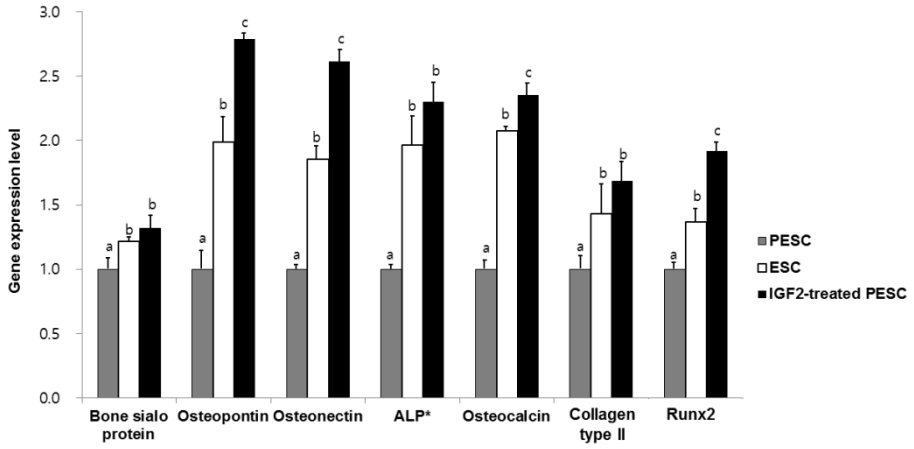

Figure 10. Osteogenic-specific gene expression in PESCs, ESCs, and IGF2treated PESCs after osteogenic induction. IGF2-treated PESCs were treated with IGF2 for the entire osteogenic induction period. ESCs were used as the control. Values with different superscripts are significantly different (a, b, c; $p < 0.05$). Expression levels of *Osteopontin* ($^{bc}p = 0.017$), *Osteonectin* ($^{bc}p = 0.021$), *Osteocalcin* ($^{bc}p = 0.042$) and *Runx2* ($^{bc}p = 0.032$) were significantly higher in IGF2-treated PESC derivatives than the other groups. ALP* is *Alkaline phosphatase*.

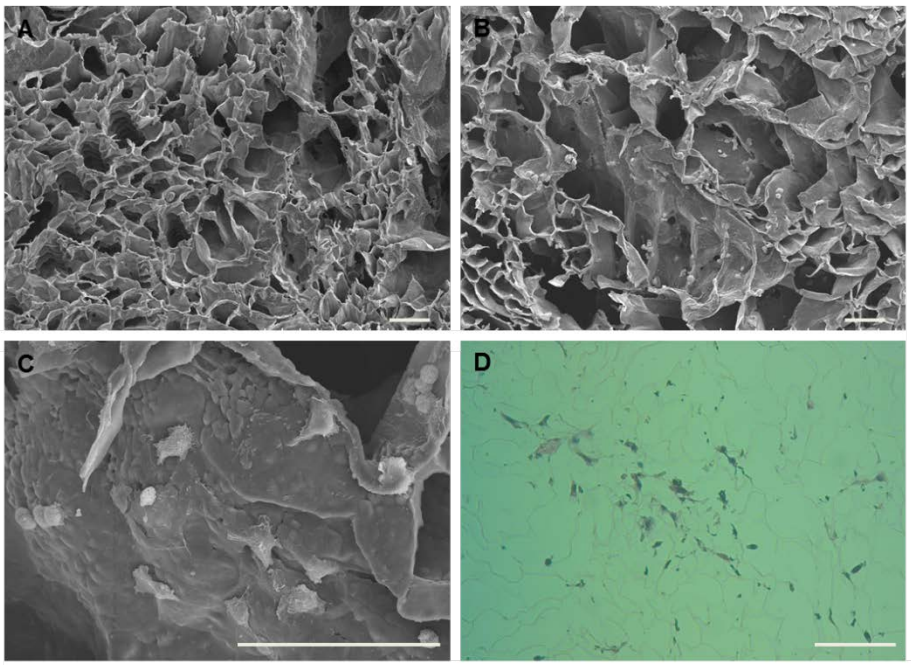

Figure 11. The morphologies of the PLLA scaffold and cultured PESC-derivatives in the PLLA. (A) Scanning electron microscope photographs of scaffold cross-sections. The prepared PLLA scaffolds were highly porous and the irregular shaped pores are interconnected. (B, C, D) Photographs of PESC-derivatives in the scaffold pores after 24 h of seeding in the PLLA by scanning electron microscopy (SEM; B, C) and hematoxylin and eosin (H&E) staining for histological evaluation (D). The SEM and H&E staining photos show that well attached and live status of the cells in the scaffold. Original magnification: X 100 (A, B); X 200 (D); X 500 (C)

After implantation of PLLA scaffolds containing IGF2-treated or IGF2-naive PESC derivatives in critical-sized calvarial bone defects, greater bone regeneration was evident in soft X-ray images in the defects treated with PLLA scaffolds containing IGF2-treated PESC derivatives than those treated with PLLA scaffolds containing IGF2-naive ones (Fig. 12). These results were confirmed by micro-CT measurements. New bone formation was about 2-fold higher in the group treated with IGF2-treated PESC derivative than in the group treated with IGF2-naive ones (Fig. 13), based on micro-CT measurements 10 weeks after implantation. Histological analyses showed new bone regeneration in the calvarial bone defect regions of defects treated with scaffolds containing IGF2-treated cells (Fig. 14). In the scaffold-only (control) group, the defect region was filled with fibrous tissues due to foreign body reactions. Hard tissue and bone regeneration were not detected in the control group. The histological features of implantation sites treated with IGF2-naive PESC derivatives were similar to those seen in the control group though small foci of dystrophic calcification were observed in some IGF2-naive PESC.

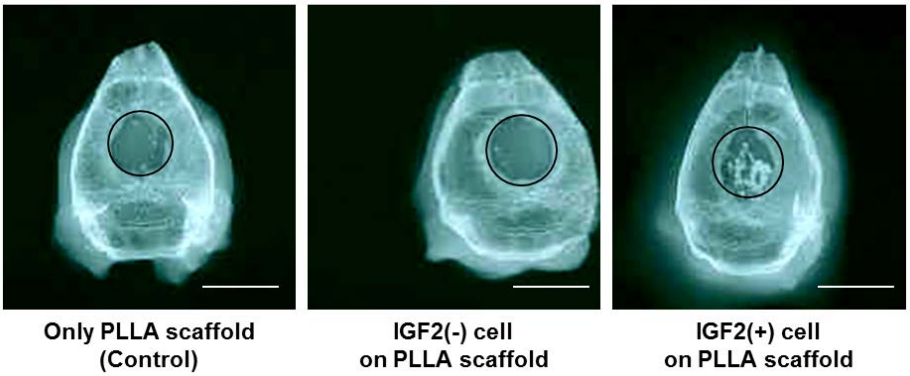

Figure 12. Soft X-ray images of the *in vivo* implantation site in critical-sized calvarial defects in mice. The circles indicate the original defect regions. The white dots within the circles may be calcification materials. (Left) Control group in which the PLLA scaffold only was implanted. (Center) Group implanted with PESCs. Before implantation, cells were induced to differentiate into an osteogenic lineage without IGF2 treatment for the entire induction period. (Right) Group implanted with IGF2-treated PESCs. Cells were treated with IGF2 for the entire induction period before implantation. More bone masses were observed in calvarial defects treated with PLLA scaffolds containing IGF2-treated cells than calvarial defects treated with scaffolds containing IGF2-naive cells. Scale bar= 5 mm.

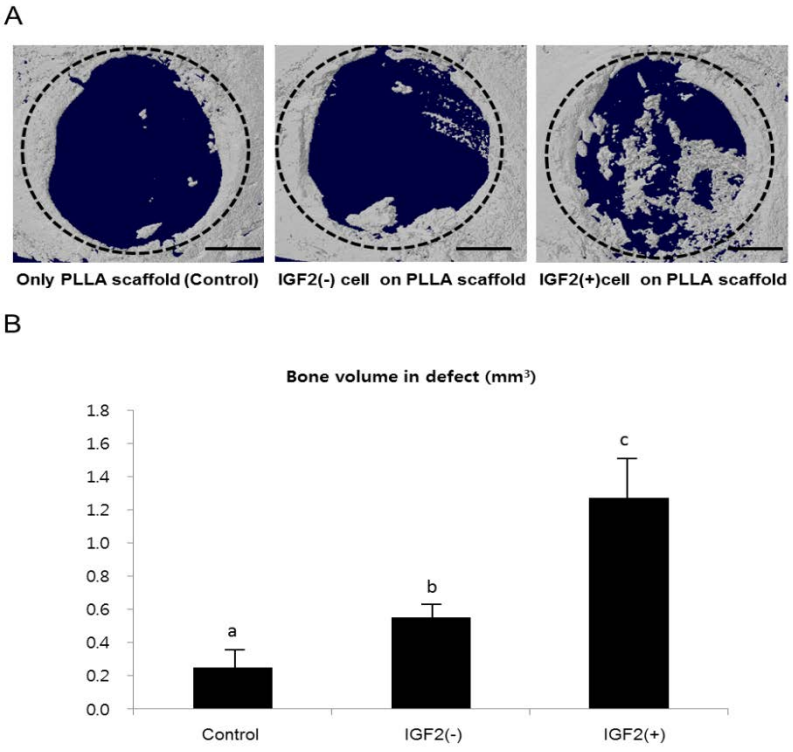

Figure 13. Micro-CT results of bone formation in critical-sized calvarial defects in mice. (A) Reconstructed micro-CT images of the scaffolds 10 weeks after implantation (Left, Control; Center, IGF2-naive PESCs; Right, IGF2-treated PESCs). The dotted circles indicate the original defects. New bone formation was about 2-fold higher in the group treated with IGF2-treated cells than in group treated with IGF2-naive cells. Scale bar= 1 mm. (B) Bone volumes of the constructs after subtracting the value of the blank control (did not receive any implant, including the scaffold). Values with different superscripts are significantly different (a, b, c; $p < 0.05$, one-way ANOVA-test). Number of samples in each group is four.

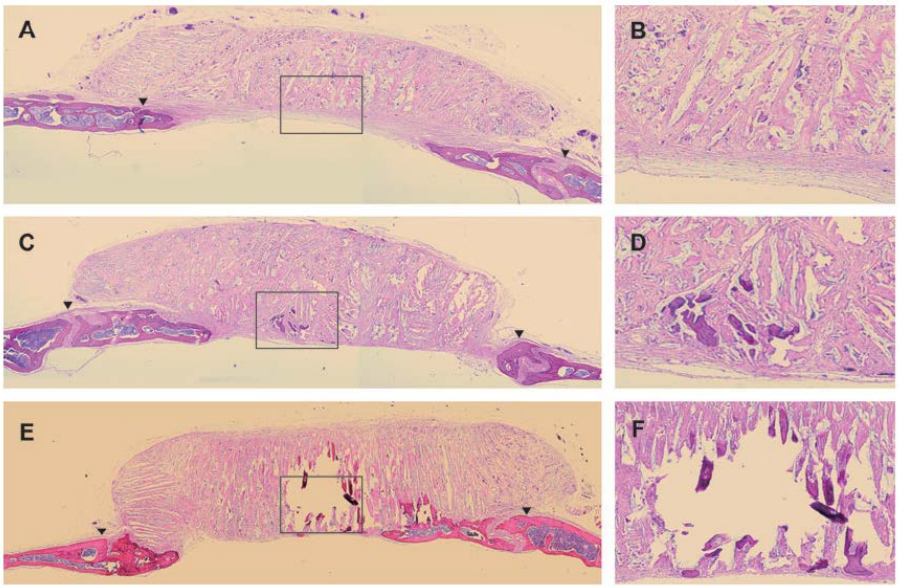

Figure 14. Histological images after H&E staining. (A, B) In the control (scaffold only), the defect region after implantation was filled with fibrous tissues because of foreign body reactions, and hard tissue and bone regeneration were not detected. (C, D) In the group treated with IGF2-naive PESC derivatives, the histological features of the implantation site were similar to those seen in the control, although small foci of dystrophic calcification were observed in some specimens (rectangular box in the middle). (E, F) In the group treated with IGF2-treated cells, new bone regeneration was clearly evident in the calvarial bone defect region (rectangular box in the middle). Arrowheads mark the site of the original defect. Original magnification: X 40 (A, C, E); X 100 (B, D, F).

3. Chondrogenic differentiation of parthenogenetic murine embryonic stem cells

Comparison of 2D and 3D culture systems for chondrogenic differentiation

Alcian blue and Safranin O staining results from the comparison of 3D and 2D culture systems are shown in Fig. 15. The regions stained with Alcian blue (Fig. 15A, B) and Safranin O (Fig. 15C, D) represent glycosaminoglycan and proteoglycan secreted by chondrogenic cells, respectively, that accumulated around the chondrogenic cells in both the 2D (Fig. 15A, C) and 3D (Fig. 15B, D) groups. The results indicate that the differentiated cell population derived from PESCs entered the chondrogenic lineage in all experimental groups. The levels of *Pax1* (p = 0.009), *Chordin-like 1* (p = 0.012), *Collagen type II* (p = 0.014), and *Aggrecan* (p = 0.007) expression were significantly higher in the 3D culture group than in the 2D culture group. The levels of *Decorin* (p = 0.093) and *MMP13* (p = 0.056) expression were not significantly different between the two groups (Fig. 16).

Optimization of IGF2 supplementation for chondrogenic differentiation in the 3D culture system

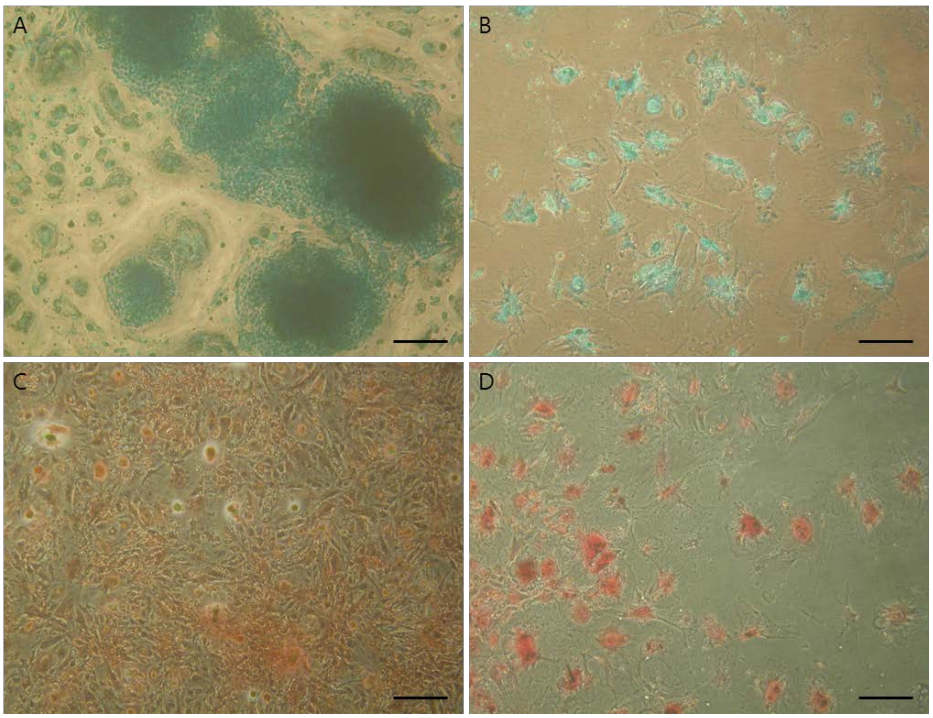

Figure 15. Alcian blue and Safranin O staining of PESC-derived chondrogenic cells induced in 2D and 3D culture systems. (A) Alcian blue staining of PESC-derivatives from 2D culture system. (B) Alcian blue staining of PESC-derivatives from the 3D culture system. (C) Safranin O staining of PESC-derivatives from the 2D culture system. (D) Safranin O staining of PESC-derivatives from the 3D culture system. The regions stained with Alcian blue and Safranin O indicates glycosaminoglycan and proteoglycan, respectively, secreted by chondrogenic cells. Scale bar= 100 μm.

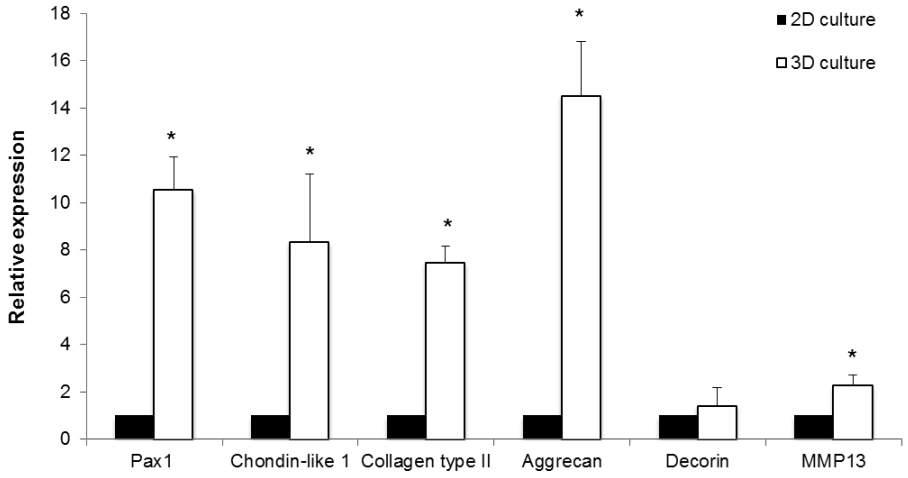

Figure 16. Chondrogenic-specific gene expression in PESC-derived chondrogenic cells induced in 2D and 3D culture systems. The extraction of mRNA and the synthesis of cDNA were performed twice, and all samples were run in triplicate to obtain technical replicates. Values with different superscripts are significantly different ($p < 0.05$, paired samples *t*-test).

Chondrogenic specific gene expression in PESC-derived chondrogenic cells after supplementation with various concentrations of exogenous IGF2 during induction in a 3D culture system and normal murine chondrocytes obtained from mouse femoral heads and condyles were analyzed by real-time qPCR. The expression levels of all chondrogenic marker genes analyzed by real-time PCR were significantly higher in the 100 ng/ml IGF2 supplementation group than in the 0, 1 and 10 ng/ml groups. The levels of expression were 7- to 35-fold higher than in the control. The expression levels of five chondrogenic marker genes in the 1 ng/ml group, excluding *MMP13*, were similar to those in the IGF2 free control. However, in the 10 ng/ml group, the expression levels of four marker genes, excluding *Collagen type II* and *Decorin* were upregulated when compared with the 0 and 1 ng/ml groups. The expression levels of pre-cartilage (*Chordin-like 1* and *Pax1*) or chondrocyte maturation marker (*Decorin*) in the 100 ng/ml IGF2 supplementation group showed similar (*Pax1*) to or significantly higher (*Chordin-like 1* and *Decorin*) than the levels in normal chondrocytes obtained from mouse femoral heads and condyles while the other marker genes (*Aggrecan*, *Collagen type II* and *MMP13*) which are normally expressed in mature chondrocytes (Fig. 17).

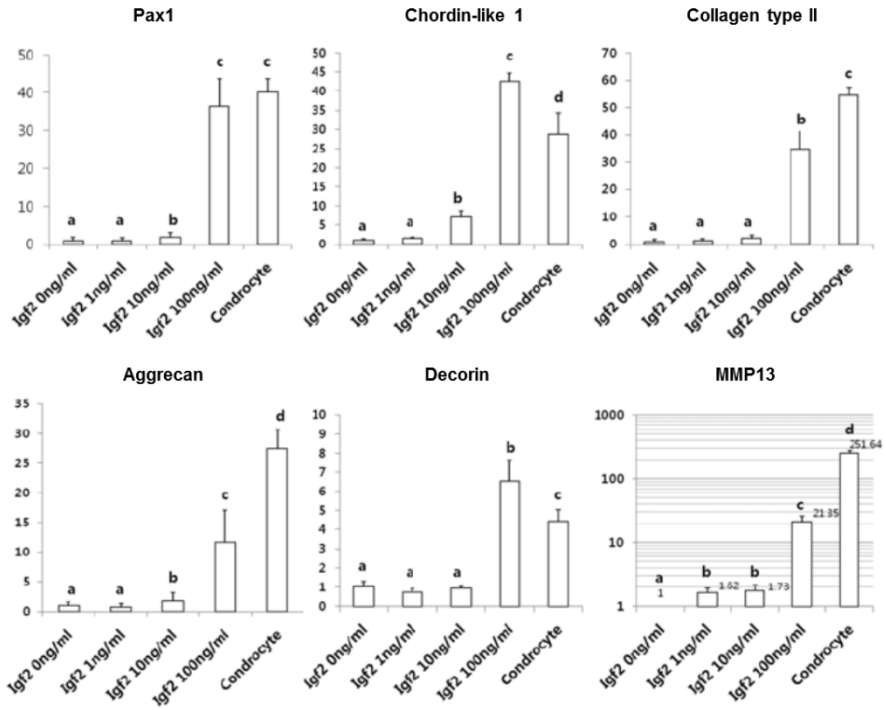

Figure 17. Chondrogenic-specific gene expression in PESC-derived chondrogenic cells after supplementation with various concentrations of exogenous IGF2 during induction in a 3D culture system and normal murine chondrocytes obtained from mouse femoral heads and condyles. mRNA extraction and cDNA synthesis were performed twice, and all samples were run in quadruplicate to obtain technical replicates. Values with different superscripts are significantly different ($p < 0.05$, paired samples *t*-test).

DISCUSSION

1. Generation of parthenogenetic murine embryonic stem cells

In the previous report, this new culture system called MTC provides an oil-free culture environment and is easy to handle, as well. In addition, the MTC system enhanced the development of pre-implantation stage murine embryos. More embryos in MTC reached the blastocyst stage with a larger number of trophectodermal cells and developed faster than those in conventional micro-drop culture. Here, as the next step, I established the ESC lines from parthenogenetic murine blastocysts produced *in vitro* and found that the embryo culture system can influence the efficiency of ESC generation.

The aim of the present study was to introduce the MTC system as a more effective way of generating PESC lines than the traditional oil-covered micro-droplet culture. After the first derivation of ESC lines from the mouse blastocysts, several standard and modified protocols were introduced. However, these protocols produce ESCs with only low efficiency in the most outbred/inbred strains and require specialized tools for embryo handling [119]. During the long process of ESC derivation, IVC of the embryos can be a critical step because obtaining qualified blastocysts can result in successful ESC production [120]. Since the ICM of the blastocysts is the source of ESC,

the ICM's condition is one of the important and conclusive factors for generating the ESC line. Although those results here did not show a significantly higher ICM outgrowth rate in MTC-derived parthenotes, I had shown that MTC system supports more blastocyst formation and more cells in the blastocyst in the previous report [51] and this might resulted in the generation of MTC-derived PESC lines in the present work.

To investigate the PESC generation potential of the MTC-derived blastocysts at molecular level, we compared the expression levels of genes related with embryonic cell growth (*Igf1, Igf2, Fgf2, and Egf*), methylation status (*Dnmt3a and Dnmt3b*), and pluripotency (*Oct4 and Nanog*), in the blastocysts from either the MTC or Drop culture systems to investigate the differences of their ESC potential at the molecular level. The IGF family is important for the early embryogenesis in mammals [48, 53]. The addition of IGFs to the culture medium increased the blastocyst rate and ICM cell number in the embryos produced *in vitro*. Other growth factors, FGF and EGF, also have important roles during the early embryogenesis [52, 121, 122]. Among the growth factor genes we analyzed, the expression levels of IGF2 were the same in both MTC and Drop. Since IGF2 is a paternally expressed imprinting gene, this result indicates that the MTC system does not affect the imprinting status in the parthenogenetic blastocysts. However, expression of *Igf1* is significantly higher in the MTC blastocyst, and this may have resulted in higher blastocyst development and ICM cell number in the embryos as previously reported [51]. DNA methylation is implicated in controlling

imprinting gene expression, X chromosome silencing, and embryonic development. It is also believed that methylation protects the genome from parasitic elements, such as transposons and viruses [55]. The murine DNMT3 family consists of two genes, *Dnmt3a* and *Dnmt3b*, which are essential for de novo methylation in murine ESC and early murine embryos but down-regulated after differentiation and expressed at low levels in adult somatic tissues [123]. Abnormal or weak expression of both DNMT3a and DNMT3b interrupts de novo DNA methylation in ESC or genome-wide de novo methylation during early mammalian development [124]. Therefore, highly expressed genes of the DNMT3 family are essential in generating ESCs [55]. DNMT3b is also expressed in mouse hematopoietic progenitor cells, spermatogonia, and during neural cell development in the murine embryo [125, 126]. Here, I found that expression of *Dnmt3a* increases significantly in the MTC group, whereas *Dnmt3b* does not. This result suggests that MTC system for embryo culture may affect the methylation status of *in vitro*-produced parthenogenetic embryos. This may be caused by de novo DNA methylation (a role of *Dnmt3a*) in ESCs. The genes related to cell pluripotency, such as OCT4 and NANOG, affect ICM quality and ESC line establishments [56]. Those genes also affect the expression of up- and down-stream genes, as well as embryonic growth and development during early embryogenesis. The parthenogenetic blastocysts in MTC showed higher *Oct4* expression than those in Drop although the number of ICM cells was not increased in MTC [51]. On the other hand, no difference was found in *Nanog*

expression between the two groups. As OCT4 (POU transcription factor, also known as Oct3 or Oct3/4) is known as an ICM-related gene, higher expression of this gene may correlate with an increased ICM number in MTC-derived blastocysts [56]. Although *Oct4* expression is known to follow *Fgf* expression in the pre-implantation embryos, an increased *Oct4* level in the MTC system may not be due to *Fgf* because there is no difference in the Fgf expression level between MTC and Drop systems [56]. Promoted expression of developmentally important genes in the MTC-derived blastocysts, such as *Oct4*, *Igf1*, and *Dnmt3a* might result in more blastocysts, faster embryonic development, and larger cell numbers in the MTC blastocysts and these should support generation of pluripotent stem cells from the parthenogenetic murine embryos.

In vivo and *in vitro* differentiation experiments showed interesting results. In general, the teratoma from ESC forms three germ layer lineage cells in the immune-deficient mouse, and this proves ESC pluripotency *in vivo*. However, in this experiment, PESCs showed only 2-germ layer (mesoderm, ectoderm) differentiation in the teratoma (Fig. 8). No endoderm tissue was observed in PESC-derived teratomas. No endoderm-specific gene expression was observed in PESC-derived EBs, as well (Fig. 7). This phenomenon may due to abnormal imprinting gene expression in PESCs. Although other reports also claimed that PESC shows restricted tissue distribution [45, 127], the recent study showed no contribution restriction in PESC chimeras [128]. Difference of the cell-lines used in each studies is may be one of the explanation as well

as the other environmental factors. Although our present data shows lower establishment rate of PESC in B6D2F1 than other groups reported [129], During the manipulation of mammalian embryos, such as *in vitro* production, intracytoplasmic sperm injection, or nuclear transfer, the oocytes or embryos exposed to an artificial or unnatural environment show poorer developmental competence than *in vivo* ones [130]. This phenomenon occurs more often in inbred or outbred strains than in F1-hybrids in the production of *in vitro* embryos and ESC line establishment [131]. After the first derivation of ESC lines from blastocysts, several standard and modified protocols were introduced. These protocols produce ESC with low efficiency in most outbred strains and require specialized tools for embryo handling [119, 132]. Specifically conditioned medium [131, 133], genetic modification of the embryo, and microdissection of the blastocyst improve mouse ESC generation [134]. Such modifications improve ESC efficiency but require specialized techniques. Because obtaining qualified blastocysts is a pre-requisite for successful ESC generation and, although many other laboratories have focused on the post-blastocyst seeding step to enhance ESC generation efficiency, our MTC system that increases the number of qualified blastocysts might result in efficient ESC generation.

In conclusion, the MTC system, involving oil-free micro-tube culture method, is an effective embryo culture method for generating PESC lines in hybrid (C57BL/6 x DBA2), and this may be due to the promoted expression of developmentally important genes, such as *Oct4*, *Igf1*, and *Dnmt3a*.

2. Osteogenic differentiation of parthenogenetic murine embryonic stem cells

ESCs have been induced to differentiate the wide variety of cell types by supplementing the culture medium with specific factors. Cultures of ESCs in defined media containing various proteins and small molecules have been used as a strategy to investigate ESC differentiation *in vitro* [135, 136]. In particular, culturing cells under defined conditions in medium containing several factors is a good strategy for discovering factors that are critical for inducing or improving specific cell lineages. I have shown previously that PESCs, which are derived from parthenogenetic blastocysts, can be induced to differentiate into an osteogenic lineage by supplementation of the culture medium with defined induction factors [45]. However, the differentiation potential of PESCs is limited compared to that of ESCs [36, 54]. The limited differentiation potential of PESCs relative to ESCs may be related to the abnormal expression of imprinting genes in PESCs. We hypothesized that the addition of a soluble factor (IGF2) that is silenced in PESCs could potentially enhance the osteogenic cell differentiation of PESCs. PESCs do not express IGF2, and these results demonstrate, the lack of endogenous IGF2 expression can be compensated for by exogenous supplementation with this factor, resulting in osteogenic cell differentiation. As mentioned earlier, IGF2 is a paternally expressed imprinting gene that is therefore not expressed in PESCs. I demonstrated using both *in vivo* and *in vitro* experiments, that IGF2

enhanced the osteogenic differentiation potential of PESCs. *In vitro* study results reveal that IGF2-treated PESC derivatives differentiated into an osteogenic cell lineage better than PESCs not exposed to IGF2. The osteoblast-specific gene expression of IGF2-treated PESC derivatives was more similar to that of ESC derivatives than IGF2-naive PESC derivatives. Bone is a mineralized connective tissue that consists mainly of collagen type I and other fiber or non-fiber matrix proteins, such as marker genes analyzed in this study and proteoglycans [137]. Osteopontin, a non-collagenous bone matrix molecule, is associated with osteogenic cell adhesion and is abundantly expressed during the early stages of osteoblast differentiation in the mouse. The expression of intermediate/late osteogenesis markers such as Runx2 (Cbfa-I), bone sialoprotein, and osteocalcin confirms the existence of a fully differentiated osteogenic cell population in addition to osteogenic progenitors [67, 138]. Therefore, *in vitro* results indicate that IGF2 promoted the osteoblastic differentiation of PESCs.

To confirm this *in vitro* result, an *in vivo* transplantation experiment was performed using a calvarial defect mouse model. We performed this *in vivo* study to determine whether the IGF2-treated PESC derivatives possessed actual bone formation capacity and could potentially be used to stimulate hard tissue regeneration. Ten weeks after cell transplantation, soft X-ray, micro-CT, and histochemistry results confirmed that the IGF2-treated PESC derivatives had greater bone regenerative potential than IGF2-naive ones. MSC are widely used in bone regeneration research. They have the capacity to express

markers of various type of tissues including muscle, nerve, bone and cartilage [99, 139]. Implantation of MSC is known to have the potential to enhance healing of bone and cartilage [104, 140]. In particular, bone regeneration result shows approximately 10~20% healing efficiency in calvarial defect murine model experiments [141, 142]. Healing efficiency in the present study is compatible to previous reports using MSC as the cell source. By the treatment of IGF2 during osteogenic induction, bone differentiating capacity of PESCs could be reached to the similar level of MSC. As shown in Fig 13, samples in all experimental groups show minor bone healing evidence around the defect region. This originates from a natural bone healing process, not from the osteogenic process of PESC derivatives. In contrast, from the natural bone healing, the sample of IGF2-treated PESC derivatives shows well defined bone regeneration from the center of scaffold which proves that the bone regeneration comes from PESC derivatives. Although there is an evidence of central bone regeneration in IGF2-naive PESC-derivatives, the regeneration efficiency is remarkably lower than IGF2-treated counterpart. Regardless of IGF2 treatment, the reason for bone regeneration majorly occurred in the center of scaffolds might be related with the way of cell seeding methods. The cells with medium tend to be placed onto the center of the scaffold by dropping with a pipette during the seeding, enabling more cells to be located in the center of the scaffold than in the peripheral part. As the result, more bone regeneration can be found in the central region of the scaffold. At present, autogenous graft is considered as the standard in

regeneration therapy protocols [143, 144]. However, this has some disadvantages such as concern for harvesting affordable amount of cells and the need for more surgeries. Application of biomaterials such as PLLA can be an alternative to autogenous graft [143]. PLLA is becoming an environmentally sustainable alternative to petro-chemical derived products due to its biodegradable characteristics and the renewable nature of its feedstock [145], in addition to its nontoxic and non-carcinogenic effects to the local tissues [90]. It has been reported that implantation of PLLA is supportive for bone regeneration in *in vivo* models [146]. However, in this study, implantation of PLLA without osteogenic cells shows very limited bone regeneration capacity when compared with the implantation results of PLLA with IGF2-treated or naive PESC-derivatives. Thus, implantation of the scaffold with cells having osteogenic potential may be still crucial for successful bone regeneration at the clinical level.

In conclusion, the results suggest that supplementation of the culture media of PESCs with exogenous IGF2 induces these cells to differentiate into an osteogenic lineage. Although there are many remarkable reports in the regenerative medical literature of successful tissue engineering using cellular supports, few of these studies investigated PESCs and their derivatives, despite the fact that by using PESCs, the ethical concerns associated with embryonic stem cell research can be avoided [147]. If osteogenic cells from PESCs could enhance bone regeneration in humans, PESCs could potentially be used to treat female patients with irreversible osteoporosis or bone loss

problems. PESCs are therefore a potentially viable alternative therapy for hard tissue regeneration and skeletal tissue repair.

3. Chondrogenic differentiation of parthenogenetic murine embryonic stem cells

In the present study, we attempted to compare the efficiency of two culture methods by measuring the levels of chondrogenic marker gene expression. Compared with conventional monolayer 2D culture, the alginate beads that are used in the 3D culture system upregulated the expression of five chondrogenic marker genes approximately 4-to 14-fold. Multi-lineage differentiation has been attempted using the 2D culture system for chondrogenic cell induction from buffalo PESCs [8]. In this report, dissociated feeder-free ESCs were cultured in induction medium for 30 days to induce chondrogenic differentiation. The cell aggregates were small and round, positive for Alcian blue staining, immunoreactive to Collagen *type II* antibody, and expressed the Collagen *type II* and SOX9 genes. However, direct-plating of EBs, which is called conventional 2D culture, did not provide a controlled seeding density and was insufficient for constructing optimal conditions for chondrogenic induction when compared with the 3D micro-mass culture system [148]. For mammalian cell culture a 3D microenvironment such as hydrogels promotes cell-to-cell interactions through secreted extracellular matrix and produces spherical cellular

morphologies. The limited space of the 3D microenvironment also suppresses the proliferation and differentiation of endothelial cells or other migratory cells from EBs, which are frequently observed in conventional 2D culture of EBs [135]. The 3D culture system is more similar to *in vivo* environments. Thus, some studies have reported distinct cellular behavior observed only in 3D culture [149]. These results showing higher expression levels of chondrogenic marker genes in a 3D culture system demonstrate the therapeutic potential of this system as it is further developed to mimic the behavior of normal chondrocytes.

In the second round of experiments, we evaluated the effect of various concentrations of exogenous IGF2 from 0 to 100 ng/ml on the *in vitro* chondrogenic differentiation of PESCs by measuring the expression levels of chondrogenic marker genes. IGF2 is an important factor for organ formation in embryonic development. However, expression of the IGF2 gene is abnormal in PESCs because it is an imprinted gene that is only expressed by the paternal genome, which is absent in parthenogenetic embryos and PESCs [72]. The inability of PESCs to completely differentiate may be related to the abnormal expression of imprinted genes such as IGF2 in PESCs. We hypothesized that the addition of soluble IGF2 could potentially enhance the chondrogenic cell differentiation of PESCs, and the results demonstrate that exogenous supplementation of IGF2 can compensate for the lack of endogenous IGF2 expression. As expected, all marker genes were highly expressed in the 100 ng/ml IGF2 supplementation group. The levels of

expression were 7- to 35- fold higher than in the control and the expression levels were similar to those observed in normal murine chondrocytes, which were derived from the femoral heads and condyles of adult mice.

According to a previous report from another research group, treatment of human chondrocytes with 100 ng/ml IGF2 enhanced the expression of the chondrogenic marker genes, *Aggrecan* and *Sox9*. *Sox9* is a pre-cartilage marker gene [111]. Treatment of human chondrocytes with IGF2 has been shown to activate the PI3K and TGF-ß pathways [111]. The P13K pathway activates the transcription of chondrogenic genes such as *Aggrecan* and *Versican* [111]. Similarly, in the present study, the levels of all six chondrogenic marker genes increased in the 100 ng/ml IGF2 supplementation group. *Chordin-like 1*, a pre-cartilage marker gene, that is primarily expressed in condensing mesenchymes and more highly expressed in chondrogenic cells than in normal chondrocytes [150]. In the present study, *Decorin*, which is upregulated during the maturation phase of cartilage development, and *Chordin-like 1* expression were increased by 1.5-fold in the 100 ng/ml IGF2 group compared to that in normal chondrocytes. In the case of another pre-cartilage marker, *Pax1*, a gene encoding a transcription factor involved in mesenchymal differentiation, the expression level in the 100 ng/ml IGF2 group was comparable to that in normal chondrocytes [103]. In contrast, expression levels of *Aggrecan* and *Collagen2*, which are components of the extracellular matrix of mature cartilage were lower in the 100 ng/ml IGF2 group compared to the levels in the normal chondrocytes [151]. In addition,

there was a remarkable decrease in the expression of *MMP13*, which is expressed in both hypertrophic chondrocytes and osteoblasts [105]. These results indicate that treatment of PESCs with the appropriate concentration of exogenous IGF2 can enhance chondrogenic differentiation, particularly in the pre or early mature stage of development.

In conclusion, a 3D culture system is more reliable for *in vitro* culturing of murine PESCs for chondrogenic differentiation. In addition, supplementation with the optimal concentration of IGF2 improves the efficiency of chondrogenic differentiation of murine PESCs in a 3D culture environment. We assume that the addition of IGF2 may re-activate genes or factors in the downstream of IGF2 gene, which only express in the paternal genome and are normally silent in PESCs, and such compensative supplementation of imprinting factor(s) can improve the efficiency of differentiation to specific lineages in monogenic stem cells.

CONCLUSION

In regenerative therapy of bone and cartilage, mesenchymal stem cells, one of the ASCs, have the potential to repair damaged tissues. MSCs have special immune-regulation factors that inhibit immune rejection of transplanted MSCs. Moreover, these cells are already used in clinical trials for the repair of damaged bones and cartilages. Although some cases have shown success in the clinical trials using MSCs, MSCs, which are isolated and cultured from human bone marrow, are rarely found in adult tissues. Moreover, the ability of isolated cells to proliferate and differentiate decreases based on the age of the related donors. Thus, transplanted MSCs from allogeneic donors increase the rate of immune rejection. To overcome these limitations, ESCs as a novel cell source have emerged and been studied for use in regenerative therapeutics. ESCs have not been used in human clinical trials but have been widely studied, and clinical results in animal models have been widely established. Nevertheless, these cells with their potential use as a cell source for bone and cartilage regeneration have several challenges as well as ethical concerns. To avoid these problems, I investigated PESCs in this thesis. Because the mammalian parthenote cannot develop to full term, the use of PESCs avoids the ethical issues associated with human ESCs. These cells are pluripotent, and if derived from an oocyte of a female patient, have the genetic information of that patient, thus preventing immune rejection responses.

Although PESCs have those advantages compared to ESCs, they also have some defects in their differentiation potential. In this thesis, I developed one of method to overcome this weakeness of PESCs. Through simple modification of a traditional differentiational method, I found a way to overcome the weak differentiatioin potential of PESCs. Considering the lack of paternal imprinting gene expression in PESCs, we selected a paternal imprinting gene that is important for osteogenic and chondrogenic differentiation. IGF2 is one of the candidates, and this gene was reported to act as cytokine in mesoderm differentiation. Based on the confirmed results, I hypothesize that the addition of the protein of imprinting gene in the induction media would affect the differentiation. This hypothesis was confirmed with an experiment. I demonstrate that it is possible to increase the differentiation efficiency by the addition of the deficient cytokine without inducing a genetic modification. These results suggest that PESCs can be used in regenerative medicine through a simple change in the traditional culture methods. Moreover, these results provide useful information for many related fields such as stem cell biology and embryo technology.

REFERENCES

[1] Donovan PJ, Gearhart J. The end of the beginning for pluripotent stem cells. Nature. 2001;414:92-7.

[2] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145-7.

[3] Mittwoch U. Parthenogenesis. J. Med. Genet. 1978;15:165-81.

[4] Bierzychudek P. Patterns in plant parthenogenesis. Experientia Supplementum. 1987;55:197-217.

[5] Lin H, Lei J, Wininger D, Nguyen MT, Khanna R, Hartmann C, et al. Multilineage potential of homozygous stem cells derived from metaphase II oocytes. Stem cells. 2003;21:152-61.

[6] Kim K, Lerou P, Yabuuchi A, Lengerke C, Ng K, West J, et al. Histocompatible embryonic stem cells by parthenogenesis. Science. 2007;315:482-6.

[7] Mai Q, Yu Y, Li T, Wang L, Chen MJ, Huang SZ, et al. Derivation of human embryonic stem cell lines from parthenogenetic blastocysts. Cell. Res. 2007;17:1008-19.

[8] Sritanaudomchai H, Pavasuthipaisit K, Kitiyanant Y, Kupradinun P, Mitalipov S, Kusamran T. Characterization and multilineage differentiation of embryonic stem cells derived from a buffalo parthenogenetic embryo. Mol. Reprod. Dev. 2007;74:1295-302.

[9] Wang S, Tang X, Niu Y, Chen H, Li B, Li T, et al. Generation and characterization of rabbit embryonic stem cells. Stem cells. 2007;25:481-9.

[10] Lane M, Gardner DK. Embryo culture medium: which is the best? Best. Pract. Res. Clin. Obstet. Gynaecol. 2007;21:83-100.

[11] Biggers JD, McGinnis LK, Lawitts JA. One-step versus two-step culture of mouse preimplantation embryos: is there a difference? Hum. Reprod. 2005;20:3376-84.

[12] Lawitts JA, Biggers JD. Optimization of mouse embryo culture media using simplex methods. J. Reprod. Fertil. 1991;91:543-56.

[13] Swain J. Embryo culture and pH. Fertil. Steril. 2011;95:e67-78.

[14] Swain JE, Cabrera L, Xu X, Smith GD. Microdrop preparation factors influence culture-media osmolality, which can impair mouse embryo preimplantation development. Reprod. Biomed. Online. 2012;24:142-7.

[15] Swain JE, Smith GD. Advances in embryo culture platforms: novel approaches to improve preimplantation embryo development through modifications of the microenvironment. Hum. Reprod. Update. 2011;17:541-57.

[16] Thouas GA, Jones GM, Trounson AO. The 'GO' system--a novel method of microculture for in vitro development of mouse zygotes to the blastocyst stage. Reproduction. 2003;126:161-9.

[17] Vajta G, Peura TT, Holm P, Paldi A, Greve T, Trounson AO, et al. New method for culture of zona-included or zona-free embryos: the Well of the Well (WOW) system. Mol. Reprod. Dev. 2000;55:256-64.

[18] Vajta G, Korosi T, Du Y, Nakata K, Ieda S, Kuwayama M, et al. The Well-of-the-Well system: an efficient approach to improve embryo development. Reprod. Biomed. Online. 2008;17:73-81.

[19] Taka M, Iwayama H, Fukui Y. Effect of the well of the well (WOW) system on in vitro culture for porcine embryos after intracytoplasmic sperm injection. J. Reprod. Dev. 2005;51:533-7.

[20] Hoelker M, Rings F, Lund Q, Ghanem N, Phatsara C, Griese J, et al. Effect of the microenvironment and embryo density on developmental characteristics and gene expression profile of bovine preimplantative embryos cultured in vitro. Reproduction. 2009;137:415-25.

[21] Sugimura S, Akai T, Somfai T, Hirayama M, Aikawa Y, Ohtake M, et al. Time-lapse cinematography-compatible polystyrene-based microwell culture system: a novel tool for tracking the development of individual bovine embryos. Biol. Reprod. 2010;83:970-8.

[22] Reik W, Walter J. Genomic imprinting: parental influence on the genome. Nat. Rev. Genet. 2001;2:21-32.

[23] Reik W, Walter J. Evolution of imprinting mechanisms: the battle of the sexes begins in the zygote. Nat. Genet. 2001;27:255-6.

[24] Martienssen RA, Colot V. DNA methylation and epigenetic inheritance in plants and filamentous fungi. Science. 2001;293:1070-4.

[25] Delaval K, Govin J, Cerqueira F, Rousseaux S, Khochbin S, Feil R. Differential histone modifications mark mouse imprinting control regions during spermatogenesis. EMBO. J. 2007;26:720-9.

[26] Feil R, Berger F. Convergent evolution of genomic imprinting in plants and mammals. Trends. Genet. 2007;23:192-9.

[27] Wood AJ, Oakey RJ. Genomic imprinting in mammals: emerging themes and established theories. PLoS. Genet. 2006;2:e147.

[28] Falls JG, Pulford DJ, Wylie AA, Jirtle RL. Genomic imprinting: implications for human disease. Am. J. Pathol. 1999;154:635-47.

[29] Jirtle RL. Genomic imprinting and cancer. Exp. Cell. Res. 1999;248:18-24.

[30] Pulford DJ, Falls JG, Killian JK, Jirtle RL. Polymorphisms, genomic imprinting and cancer susceptibility. Mutat. Res. 1999;436:59-67.

[31] Mann JR, Lovell-Badge RH. Inviability of parthenogenones is determined by pronuclei, not egg cytoplasm. Nature. 1984;310:66-7.

[32] McGrath J, Solter D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell. 1984;37:179-83.

[33] Ogawa H, Ono Y, Shimozawa N, Sotomaru Y, Katsuzawa Y, Hiura H, et al. Disruption of imprinting in cloned mouse fetuses from embryonic stem cells. Reproduction. 2003;126:549-57.

[34] Robertson EJ. Derivation and maintenance of embryonic stem cell cultures. Methods. Mol. Biol. 1997;75:173-84.

[35] Valdimarsdottir G, Mummery C. Functions of the TGFbeta superfamily in human embryonic stem cells. APMIS. 2005;113:773-89.

[36] Morali OG, Jouneau A, McLaughlin KJ, Thiery JP, Larue L. IGF-II promotes mesoderm formation. Dev. Biol. 2000;227:133-45.

[37] Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, et al. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med. 2000;6:88-95.

[38] Woll NL, Bronson SK. Analysis of embryonic stem cell-derived osteogenic cultures. Methods. Mol. Biol. 2006;330:149-59.

[39] Woll NL, Heaney JD, Bronson SK. Osteogenic nodule formation from single embryonic stem cell-derived progenitors. Stem cells. Dev. 2006;15:865-79.

[40] Bielby RC, Pryce RS, Hench LL, Polak JM. Enhanced derivation of osteogenic cells from murine embryonic stem cells after treatment with ionic dissolution products of 58S bioactive sol-gel glass. Tissue. Eng. Part A. 2005;11:479-88.

[41] Buttery LD, Bourne S, Xynos JD, Wood H, Hughes FJ, Hughes SP, et al. Differentiation of osteoblasts and in vitro bone formation from murine embryonic stem cells. Tissue. Eng. Part A. 2001;7:89-99.

[42] Warotayanont R, Frenkel B, Snead ML, Zhou Y. Leucine-rich amelogenin peptide induces osteogenesis by activation of the Wnt pathway. Biochem. Biophys. Res. Commun. 2009;387:558-63.

[43] Tian XF, Heng BC, Ge Z, Lu K, Rufaihah AJ, Fan VT, et al. Comparison of osteogenesis of human embryonic stem cells within 2D and 3D culture systems. Scand. J. Clin. Lab. Invest. 2008;68:58-67.

[44] Choi YJ, Kang H, Sung J, Park S, Hong SD, Min BM, et al. Promoted expression of Igf-1, Dnmt3a and Oct-4 in the parthenogenetic murine

blastocysts developed in an oil-free microtube culture system may support stem cell generation. Tissue. Eng. Regen. Med. 2011;8:78-86.

[45] Allen ND, Barton SC, Hilton K, Norris ML, Surani MA. A functional analysis of imprinting in parthenogenetic embryonic stem cells. Development. 1994;120:1473-82.

[46] Cibelli JB, Grant KA, Chapman KB, Cunniff K, Worst T, Green HL, et al. Parthenogenetic stem cells in nonhuman primates. Science. 2002;295:819.

[47] Wolf DP, Kuo HC, Pau KY, Lester L. Progress with nonhuman primate embryonic stem cells. Biol. Reprod. 2004;71:1766-71.

[48] Revazova ES, Turovets NA, Kochetkova OD, Agapova LS, Sebastian JL, Pryzhkova MV, et al. HLA homozygous stem cell lines derived from human parthenogenetic blastocysts. Cloning. Stem Cells. 2008;10:11-24.

[49] Liu J, Schoonjans L, Tielens S, Speleman F, Cornelissen M, De Sutter P, et al. Culturing in vitro produced blastocysts in sequential media promotes ES cell derivation. Mol. Reprod. Dev. 2006;73:1017-21.

[50] Pera MF, Reubinoff B, Trounson A. Human embryonic stem cells. J. Cell. Sci. 2000;113 (Pt 1):5-10.

[51] Roh S, Choi YJ, Min BM. A novel microtube culture system that enhances the in vitro development of parthenogenetic murine embryos. Theriogenology. 2008;69:262-7.

[52] Terada A, Minoura H, Toyoda N. Effects of epidermal growth factor on preimplantation mouse embryos. J Assist. Reprod. Genet. 1997;14:404-11.

[53] Harvey MB, Kaye PL. Insulin-like growth factor-1 stimulates growth of mouse preimplantation embryos in vitro. Mol. Reprod. Dev. 1992;31:195-9.

[54] Feil R, Walter J, Allen ND, Reik W. Developmental control of allelic methylation in the imprinted mouse Igf2 and H19 genes. Development. 1994;120:2933-43.

[55] Vassena R, Dee Schramm R, Latham KE. Species-dependent expression patterns of DNA methyltransferase genes in mammalian oocytes and preimplantation embryos. Mol. Reprod. Dev. 2005;72:430-6.

[56] Buehr M, Nichols J, Stenhouse F, Mountford P, Greenhalgh CJ, Kantachuvesiri S, et al. Rapid loss of Oct-4 and pluripotency in cultured rodent blastocysts and derivative cell lines. Biol. Reprod. 2003;68:222-9.

[57] Cavaleri F, Scholer HR. Nanog: a new recruit to the embryonic stem cell orchestra. Cell. 2003;113:551-2.

[58] Kang H, Sung J, Jung HM, Woo KM, Hong SD, Roh S. Insulin-Like Growth Factor 2 Promotes Osteogenic Cell Differentiation in the Parthenogenetic Murine Embryonic Stem Cells. Tissue. Eng. Part A. 2011.

[59] Damien CJ, Parsons JR. Bone graft and bone graft substitutes: a review of current technology and applications. J. Appl. Biomater. 1991;2:187-208.

[60] Desbaillets I, Ziegler U, Groscurth P, Gassmann M. Embryoid bodies: an in vitro model of mouse embryogenesis. Exp. Physiol. 2000;85:645-51.

[61] Ling V, Neben S. In vitro differentiation of embryonic stem cells: immunophenotypic analysis of cultured embryoid bodies. J. Cell. Physiol. 1997;171:104-15.

[62] Boheler KR, Czyz J, Tweedie D, Yang HT, Anisimov SV, Wobus AM. Differentiation of pluripotent embryonic stem cells into cardiomyocytes. Circ. Res. 2002;91:189-201.

[63] Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T, et al. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. Nature. 2000;408:92-6.

[64] Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J.Cell. Biochem. 1997;64:295-312.

[65] Caplan DJ, Weintraub JA. Factors related to loss of root canal filled teeth. J. Public. Health. Dent. 1997;57:31-9.

[66] Quarto R, Thomas D, Liang CT. Bone progenitor cell deficits and the age-associated decline in bone repair capacity. Calcif. Tissue. Int. 1995;56:123-9.

[67] Bourne S, Polak JM, Hughes SP, Buttery LD. Osteogenic differentiation of mouse embryonic stem cells: differential gene expression analysis by cDNA microarray and purification of osteoblasts by cadherin-11 magnetically activated cell sorting. Tissue. Eng. Part A. 2004;10:796-806.

[68] Kohlmeier M, Saupe J, Schaefer K, Asmus G. Bone fracture history and prospective bone fracture risk of hemodialysis patients are related to apolipoprotein E genotype. Calcif. Tissue. Int. 1998;62:278-81.

[69] Vrana KE, Hipp JD, Goss AM, McCool BA, Riddle DR, Walker SJ, et al. Nonhuman primate parthenogenetic stem cells. Proc. Natl. Acad. Sci. USA. 2003;100 Suppl 1:11911-6.

[70] Drukker M. Recent advancements towards the derivation of immune-compatible patient-specific human embryonic stem cell lines. Semin. Immunol. 2008;20:123-9.

[71] Tam PP, Behringer RR. Mouse gastrulation: the formation of a mammalian body plan. Mech. Dev. 1997;68:3-25.

[72] Burns JL, Hassan AB. Cell survival and proliferation are modified by insulin-like growth factor 2 between days 9 and 10 of mouse gestation. Development. 2001;128:3819-30.

[73] Stewart CE, James PL, Fant ME, Rotwein P. Overexpression of insulin-like growth factor-II induces accelerated myoblast differentiation. J. Cell. Physiol. 1996;169:23-32.

[74] Coolican SA, Samuel DS, Ewton DZ, McWade FJ, Florini JR. The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. J. Biol. Chem. 1997;272:6653-62.

[75] Hill PA, Reynolds JJ, Meikle MC. Osteoblasts mediate insulin-like growth factor-I and -II stimulation of osteoclast formation and function. Endocrinology. 1995;136:124-31.

[76] Mochizuki H, Hakeda Y, Wakatsuki N, Usui N, Akashi S, Sato T, et al. Insulin-like growth factor-I supports formation and activation of osteoclasts. Endocrinology. 1992;131:1075-80.

[77] Constancia M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, et al. Placental-specific IGF-II is a major modulator of placental and fetal growth. Nature. 2002;417:945-8.

[78] Carter AM, Nygard K, Mazzuca DM, Han VK. The expression of insulin-like growth factor and insulin-like growth factor binding protein mRNAs in mouse placenta. Placenta. 2006;27:278-90.

[79] Baker J, Liu JP, Robertson EJ, Efstratiadis A. Role of insulin-like growth factors in embryonic and postnatal growth. Cell. 1993;75:73-82.

[80] Lee JE, Pintar J, Efstratiadis A. Pattern of the insulin-like growth factor II gene expression during early mouse embryogenesis. Development. 1990;110:151-9.

[81] DeChiara TM, Efstratiadis A, Robertson EJ. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. Nature. 1990;345:78-80.

[82] Ludwig T, Eggenschwiler J, Fisher P, D'Ercole AJ, Davenport ML, Efstratiadis A. Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds. Dev.Biol. 1996;177:517-35.

[83] Han VK, D'Ercole AJ, Lund PK. Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus. Science. 1987;236:193-7.

[84] Gray A, Tam AW, Dull TJ, Hayflick J, Pintar J, Cavenee WK, et al. Tissue-specific and developmentally regulated transcription of the insulin-like growth factor 2 gene. DNA. 1987;6:283-95.

[85] Amaral AC, Marques AF, Munoz JE, Bocca AL, Simioni AR, Tedesco AC, et al. Poly(lactic acid-glycolic acid) nanoparticles markedly improve immunological protection provided by peptide P10 against murine paracoccidioidomycosis. Br. J. Pharmacol. 2010;159:1126-32.

[86] Huang J, Zhang L, Chu B, Peng X, Tang S. Repair of bone defect in caprine tibia using a laminated scaffold with bone marrow stromal cells loaded poly (L-lactic acid)/beta-tricalcium phosphate. Artif. Organs. 2011;35:49-57.

[87] Eisenbrey JR, Burstein OM, Kambhampati R, Forsberg F, Liu JB, Wheatley MA. Development and optimization of a doxorubicin loaded poly(lactic acid) contrast agent for ultrasound directed drug delivery. J. Control. Release. 2010;143:38-44.

[88] Gould L, Trope G, Cheng YL, Heathcote JG, Sheardown H, Rootman D, et al. Fifty:fifty poly (DL glycolic acid-lactic acid) copolymer as a drug delivery system for 5-fluorouracil: a histopathological evaluation. Can. J. Ophthalmol. 1994;29:168-71.

[89] Rasal RM, Hirt DE. Micropatterning of covalently attached biotin on poly(lactic acid) film surfaces. Macromol. Biosci. 2009;9:989-96.

[90] Rasal RM, Bohannon BG, Hirt DE. Effect of the photoreaction solvent on surface and bulk properties of poly(lactic acid) and poly(hydroxyalkanoate) films. J. Biomed. Mater. Res. B. Appl. Biomater. 2008;85:564-72.

[91] Kang H, Jeong YD, Verma P, Roh S. Induced Chondrogenic Differentiation of Parthenogenetic Murine Embryonic Stem Cells by Insulin-like Growth Factor 2 Treatment in a Three-Dimensional Culture Environment. Tissue. Eng. Regen. Med. 2013;10:380-7.

[92] Hardingham T, Tew S, Murdoch A. Tissue engineering: chondrocytes and cartilage. Arthritis. Res. 2002;4 Suppl 3:S63-8.

[93] Buckwalter JA, Mankin HJ. Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. Instr. Course. Lect. 1998;47:487-504.

[94] Buckwalter JA. Articular cartilage injuries. Clin. Orthop. Relat. Res. 2002:21-37.

[95] Wakitani S, Kimura T, Hirooka A, Ochi T, Yoneda M, Yasui N, et al. Repair of rabbit articular surfaces with allograft chondrocytes embedded in collagen gel. J. Bone. Joint. Surg. Br. 1989;71:74-80.

[96] Dozin B, Malpeli M, Camardella L, Cancedda R, Pietrangelo A. Response of young, aged and osteoarthritic human articular chondrocytes to inflammatory cytokines: molecular and cellular aspects. Matrix. Biol. 2002;21:449-59.

[97] Caplan AI. Mesenchymal stem cells. J. Orthop. Res. 1991;9:641-50.

[98] D'Ippolito G, Schiller PC, Ricordi C, Roos BA, Howard GA. Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow. J. Bone. Miner. Res. 1999;14:1115-22.

[99] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143-7.

[100] Heng BC, Cao T, Haider HK, Wang DZ, Sim EK, Ng SC. An overview and synopsis of techniques for directing stem cell differentiation in vitro. Cell. Tissue. Res. 2004;315:291-303.

[101] Bradley JA, Bolton EM, Pedersen RA. Stem cell medicine encounters the immune system. Nat. Rev. Immun. 2002;2:859-71.

[102] Rougier N, Werb Z. Minireview: Parthenogenesis in mammals. Mol. Reprod. Dev. 2001;59:468-74.

[103] Kramer J, Hegert C, Hargus G, Rohwedel J. Mouse ES cell lines show a variable degree of chondrogenic differentiation in vitro. Cell. Biol. Int. 2005;29:139-46.

[104] Bruder SP, Jaiswal N, Ricalton NS, Mosca JD, Kraus KH, Kadiyala S. Mesenchymal stem cells in osteobiology and applied bone regeneration. Clin. Orthop. Relat. Res. 1998:S247-56.

[105] zur Nieden NI, Kempka G, Rancourt DE, Ahr HJ. Induction of chondro-, osteo- and adipogenesis in embryonic stem cells by bone morphogenetic protein-2: effect of cofactors on differentiating lineages. BMC. Dev. Biol. 2005;5:1.

[106] Humbel RE. Insulin-like growth factors I and II. Eur. J. Biochem. 1990;190:445-62.

[107] Denley A, Cosgrove LJ, Booker GW, Wallace JC, Forbes BE. Molecular interactions of the IGF system. Cytokine. Growth Factor. Rev. 2005;16:421-39.

[108] Starkman BG, Cravero JD, Delcarlo M, Loeser RF. IGF-I stimulation of proteoglycan synthesis by chondrocytes requires activation of the PI 3-kinase pathway but not ERK MAPK. Biochem. J. 2005;389:723-9.

[109] Tucci M, Nygard K, Tanswell BV, Farber HW, Hill DJ, Han VK. Modulation of insulin-like growth factor (IGF) and IGF binding protein biosynthesis by hypoxia in cultured vascular endothelial cells. J. Endocrinol. 1998;157:13-24.

[110] Kim MS, Hwang NS, Lee J, Kim TK, Leong K, Shamblott MJ, et al. Musculoskeletal differentiation of cells derived from human embryonic germ cells. Stem cells. 2005;23:113-23.

[111] Hamamura K, Zhang P, Yokota H. IGF2-driven PI3 kinase and TGFbeta signaling pathways in chondrogenesis. Cell. Biol. Int. 2008;32:1238-46.

[112] Eggenschwiler J, Ludwig T, Fisher P, Leighton PA, Tilghman SM, Efstratiadis A. Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features of the Beckwith-Wiedemann and Simpson-Golabi-Behmel syndromes. Genes. Dev. 1997;11:3128-42.

[113] Kang H, Sung J, Jung HM, Woo KM, Hong SD, Roh S. Insulin-like growth factor 2 promotes osteogenic cell differentiation in the

parthenogenetic murine embryonic stem cells. Tissue. Engineering. Part A. 2012;18:331-41.

[114] Gong SP, Kim H, Lee EJ, Lee ST, Moon S, Lee HJ, et al. Change in gene expression of mouse embryonic stem cells derived from parthenogenetic activation. Hum. Reprod. 2009;24:805-14.

[115] Ragina NP, Schlosser K, Knott JG, Senagore PK, Swiatek PJ, Chang EA, et al. Downregulation of H19 improves the differentiation potential of mouse parthenogenetic embryonic stem cells. Stem cells. Dev. 2012;21:1134-44.

[116] Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J. Reprod. Fertil. 1989;86:679-88.

[117] Zhang R, Ma PX. Poly(alpha-hydroxyl acids)/hydroxyapatite porous composites for bone-tissue engineering. I. Preparation and morphology. J. Biomed. Mater. Res. 1999;44:446-55.

[118] Gosset M, Berenbaum F, Thirion S, Jacques C. Primary culture and phenotyping of murine chondrocytes. Nat. Protoc. 2008;3:1253-60.

[119] Bryja V, Bonilla S, Cajanek L, Parish CL, Schwartz CM, Luo Y, et al. An efficient method for the derivation of mouse embryonic stem cells. Stem cells. 2006;24:844-9.

[120] Bryja V, Bonilla S, Arenas E. Derivation of mouse embryonic stem cells. Nat. Protoc. 2006;1:2082-7.

[121] Colver RM, Howe AM, McDonough PG, Boldt J. Influence of growth factors in defined culture medium on in vitro development of mouse embryos. Fertil. Steril. 1991;55:194-9.

[122] Wood SA, Kaye PL. Effects of epidermal growth factor on preimplantation mouse embryos. J. Reprod. Fertil. 1989;85:575-82.

[123] Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 1999;99:247-57.

[124] Zvetkova I, Apedaile A, Ramsahoye B, Mermoud JE, Crompton LA, John R, et al. Global hypomethylation of the genome in XX embryonic stem cells. Nat. Genet. 2005;37:1274-9.

[125] Watanabe D, Suetake I, Tajima S, Hanaoka K. Expression of Dnmt3b in mouse hematopoietic progenitor cells and spermatogonia at specific stages. GEP. 2004;5:43-9.

[126] Watanabe D, Uchiyama K, Hanaoka K. Transition of mouse de novo methyltransferases expression from Dnmt3b to Dnmt3a during neural progenitor cell development. Neuroscience. 2006;142:727-37.

[127] Hikichi T, Wakayama S, Mizutani E, Takashima Y, Kishigami S, Van Thuan N, et al. Differentiation potential of parthenogenetic embryonic stem cells is improved by nuclear transfer. Stem cells. 2007;25:46-53.

[128] Onodera Y, Teramura T, Ozawa M, Takehara T, Mitani T, Anzai M, et al. Differentiation diversity of mouse parthenogenetic embryonic stem cells in chimeric mice. Theriogenology. 2010;74:135-45.

[129] Sung LY, Chang CC, Amano T, Lin CJ, Amano M, Treaster SB, et al. Efficient derivation of embryonic stem cells from nuclear transfer and parthenogenetic embryos derived from cryopreserved oocytes. Cell. Reprogram. 2010;12:203-11.

[130] Sakai RR, Tamashiro KL, Yamazaki Y, Yanagimachi R. Cloning and assisted reproductive techniques: influence on early development and adult phenotype. Birth. Defects. Res. Part C. 2005;75:151-62.

[131] Schoonjans L, Kreemers V, Danloy S, Moreadith RW, Laroche Y, Collen D. Improved generation of germline-competent embryonic stem cell lines from inbred mouse strains. Stem cells. 2003;21:90-7.

[132] Ouhibi N, Sullivan NF, English J, Colledge WH, Evans MJ, Clarke NJ. Initial culture behaviour of rat blastocysts on selected feeder cell lines. Mol. Reprod. Dev. 1995;40:311-24.

[133] Cheng J, Dutra A, Takesono A, Garrett-Beal L, Schwartzberg PL. Improved generation of C57BL/6J mouse embryonic stem cells in a defined serum-free media. Genesis. 2004;39:100-4.

[134] McWhir J, Schnieke AE, Ansell R, Wallace H, Colman A, Scott AR, et al. Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a non-permissive genetic background. Nat. Genet. 1996;14:223-6.

[135] Hwang YS, Polak JM, Mantalaris A. In vitro direct chondrogenesis of murine embryonic stem cells by bypassing embryoid body formation. Stem cells. Dev. 2008;17:971-8.

[136] Guochun G, Deborah F, Caroline MD, Robert K. Direct and progressive differentiation of human embryonic stem cells into the chondrogenic lineage. J. Cell. Physiol. 2010;4:664-71.

[137] Casser-Bette M, Murray AB, Closs EI, Erfle V, Schmidt J. Bone formation by osteoblast-like cells in a three-dimensional cell culture. Calcif. Tissue. Int. 1990;46:46-56.

[138] Bobryshev YV, Killingsworth MC, Lord RS. Spatial distribution of osteoblast-specific transcription factor Cbfa1 and bone formation in atherosclerotic arteries. Cell. Tissue. Res. 2008;333:225-35.

[139] Sekiya I, Vuoristo JT, Larson BL, Prockop DJ. In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. Proc. Natl. Acad. Sci. USA. 2002;99:4397-402.

[140] Wakitani S, Goto T, Pineda SJ, Young RG, Mansour JM, Caplan AI, et al. Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. J. Bone. Joint. Surg. Am. 1994;76:579-92.

[141] Koob S, Torio-Padron N, Stark GB, Hannig C, Stankovic Z, Finkenzeller G. Bone formation and neovascularization mediated by mesenchymal stem cells and endothelial cells in critical-sized calvarial defects. Tissue. Eng. Part A. 2011;17:311-21.

[142] Nakahara H, Misawa H, Hayashi T, Kondo E, Yuasa T, Kubota Y, et al. Bone repair by transplantation of hTERT-immortalized human mesenchymal stem cells in mice. Transplantation. 2009;88:346-53.

[143] Becker W, Becker BE, Caffesse R. A comparison of demineralized freeze-dried bone and autologous bone to induce bone formation in human extraction sockets. J. Periodontol. 1994;65:1128-33.

[144] Becker W, Dahlin C, Becker BE, Lekholm U, van Steenberghe D, Higuchi K, et al. The use of e-PTFE barrier membranes for bone promotion around titanium implants placed into extraction sockets: a prospective multicenter study. Int. J. Oral. Maxillofac. Implants. 1994;9:31-40.

[145] Antunes JC, Oliveira JM, Reis RL, Soria JM, Gomez-Ribelles JL, Mano JF. Novel poly(L-lactic acid)/hyaluronic acid macroporous hybrid scaffolds: characterization and assessment of cytotoxicity. J. Biomed. Mater. Res. A. 2010;94:856-69.

[146] Rimondini L, Nicoli-Aldini N, Fini M, Guzzardella G, Tschon M, Giardino R. In vivo experimental study on bone regeneration in critical bone defects using an injectable biodegradable PLA/PGA copolymer. Oral. Surg. Oral. Med. Oral. Pathol. Oral. Radiol. Endod. 2005;99:148-54.

[147] Daar AS, Greenwood HL. A proposed definition of regenerative medicine. J. Tissue. Eng. Regen Med. 2007;1:179-84.

[148] Toh WS, Yang Z, Liu H, Heng BC, Lee EH, Cao T. Effects of culture conditions and bone morphogenetic protein 2 on extent of chondrogenesis from human embryonic stem cells. Stem cells. 2007;25:950-60.

[149] Schmeichel KL, Bissell MJ. Modeling tissue-specific signaling and organ function in three dimensions. J. Cell. Sci. 2003;116:2377-88.

[150] Nakayama N, Duryea D, Manoukian R, Chow G, Han CY. Macroscopic cartilage formation with embryonic stem-cell-derived mesodermal progenitor cells. J. Cell. Sci. 2003;116:2015-28.

[151] Chambers MG, Kuffner T, Cowan SK, Cheah KS, Mason RM. Expression of collagen and aggrecan genes in normal and osteoarthritic murine knee joints. Osteoarthritis. Cartilage. 2002;10:51-61.

국 문 초 록

마우스 단위발생 배아줄기세포주의 확립과 골모세포 및 연골모세포로의 분화

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골조직 손상을 치료하는 분야는 정형외과 및 치과연구에서 매우 중요한 영역이다. 전분화능을 가진 배아줄기세포는 모든 세포로의 분화가 가능하기 때문에 골조직 재생의학분야에서 좋은 세포원으로 연구되어 왔다. 본 연구에서는 단위발생 배아줄기세포를 이용하여, 이전에 알려진 골조직 분화기법에 변화를 주어 분화 효율을 높이고자 연구를 진행하였다. 단위발생배아 배양은 새로운 배양법인 micro-tube 배양기법을 이용하였다. 본 배양기법을 통해 생산된 단위발생배아는 줄기세포주 생산에 높은 효율을 보였으며, 유전자발현 분석을 통하여 관련유전자 중 배아의 성장과 관련이 있는 *Igf1* 및 전분화능과 관련된 *Oct4* 의 발현이 증가한 것을 확인할 수 있었다. 제작된 단위발생배아줄기세포는 전분화능을 가지면서도 배반포 이후 발생이 불가능한 점 때문에 배아줄기세포가 가지는 단점 인 윤리적 문제를 극복할 수 있어 전분화능 줄기세로를 이용한 재생의학연구에 있어서 좋은

세포원으로 알려져 있다. 그러나, 부계각인유전자가 발현하지 않는 것으로 인하여 분화능력은 매우 제한적인 것으로 알려져 있다. 따라서, 본 연구에서는 중요한 부계각인유전자의 유사발현을 유도할 경우, 단위발생배아줄기세포의 분화효율이 높아질 것으로 가정하였다. 부계각인유전자 중 배아의 성장에 중요한 것으로 알려진 Insulin like growth factor *2* (IGF2)를 분화 시 배양액에 첨가함으로써 분화효율에 변화를 줄 수 있는 지 확인하였다. 실험결과 IGF2 를 분화유도 배양액에 첨가하는 것 만으로 유전자발현의 보상효과를 유발하여 단위발생배아줄기세포의 골모세포 및 연골모세포로의 분화효율이 증진함을 확인하였다. 골모세포로의 분화에서는 분화유도 용액에 IGF2 를 첨가한 결과, 분화관련 유전자 중 *osteocalcin, osteopontin, osteonectin, alkaline phosphatase* 의 발현이 증가함을 확인하였다. 두경부 골조직 손상 마우스 모델을 이용한 실험에서는 체내 골조직 재생효율이 높아지는 것을 확인하였다. 연골모세포로의 분화에서도 유사한 결과를 확인하였는데 연골분화유도 용액에 IGF2 를 첨가한 결과, *decorin, chordin-like 1, pax1, aggrecan, collagen type II* 와 *MMP13* 의 발현이 증가함을 확인함으로써, IGF2 의 배양액 내 첨가는 분화효율에 효과적임을 확인하였다. 이러한 결과를 종합해 하여, 이번 연구에서는 단위발생배아줄기세포의 세포주 확립에 새로운 배아배양법이 유효함을 확인하였고 부계각인인자인 IGF2 의 배양액 내에 첨가가 단위발생 배아줄기세포주의 특정계통으로의 분화효율을 높일 수 있음을 확인하였다.

주요어: 배아배양, 단위발생, 단위발생배아줄기세포, 각인유전자, 골모세포, 연골모세포

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