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ATDC5: An Excellent In Vitro Model Cell Line for Skeletal Development

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ABSTRACT

The ATDC5 cell line is derived from mouse teratocarcinoma cells and characterized as a chondrogenic cell line which goes through a sequential process analogy to chondrocyte differentiation. Thus, it is regarded as a promising in vitro model to study the factors that influence cell behaviors during chondrogenesis. It also provides insights in exploring signaling pathways related to skeletal development as well as interactions with innovative materials. To date, over 200 studies have utilized ATDC5 to obtain lots of significant findings. In this review, we summarized the literature of ATDC5 related studies and emphasized the application of ATDC5 in chondrogenesis. In addition, the general introduction of ATDC5 including its derivation and characterization is covered in this article. J. Cell. Biochem. 114: 1223–1229, 2013.

KEY WORDS: ATDC5; CHONDROGENESIS; ENDOCHONDRAL OSSIFICATION

Cartilage is an avascular, aneural, and alymphatic tissue that has a very limited capability to self-regenerate after damaged by injuries or degenerative diseases. In recent decades, novel tissue engineering strategies for cartilage repair have attracted more and more attentions and a large number of studies have been carried out on. Cartilage tissue engineering aims to repair or regenerate damaged cartilage mainly with three-dimensional (3D) cell-laden scaffolds and suitable biological molecules [Chung and Burdick, 2008]. The factors involved in cartilage repair are so complicated that no ideal means has been acknowledged so far. Therefore, it is crucial to figure out the mechanisms of chondrogenesis, including the key pathways trigged by biological molecules and the ideal biomimetic microenvironment in 3D scaffolds for chondrogenesis, etc.

To this end, it has prompted the search for stable model cell line with such advantages as infinite and rapid proliferation along with its homogeneity. Many cell lines, such as C3H10T1/2 [Ogasawara et al., 2012], C2C12 [Rajan et al., 2012], MG63 [Tsai et al., 2012], etc., have been widely used to study the differentiation of myoblast and osteoblast. Compared to primary cells that are influenced by the source and passage number, these model cell lines are more homogeneous and consistent. Thus they are more suitable for study of the molecular mechanism. ATDC5 cells, first introduced in 1990, appear to be a useful tool in studying the differentiation of chondrocytes as well as exploring mechanistic pathways [Atsumi et al., 1990].

Within this review, the derivation and characterization of ATDC5 cells will be briefly introduced, followed by a detailed introduction of in vitro studies using ATDC5 cells.

BRIEF OVERVIEW OF ATDC5 CELLS

To date, the ATDC5 cell line has been used as an in vitro model in nearly 300 studies which have been published, according to the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/).

Atsumi et al. [1990] reported that ATDC5 cells, isolated from mouse teratocarcinoma fibroblastic cells, could exhibit chondrogenic differentiation at a high frequency, compared with other established cell lines (C3H10T1/2 and RJC3.1). Subsequently, in

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vitro studies by Shukunami et al. [1996, 1997] demonstrated that ATDC5 cells could undergo cellular condensation and sequential chondrogenic differentiation with characterization of proteoglycan synthesis and type II collagen expression when treated with insulin. With the increasing studies of ADTC5 cells, more and more evidence was shown that this cell line has properties to reproduce multi-steps of chondrocyte differentiation [Suzuki, 1996; Shukunami et al., 1998; Tare et al., 2005]. During the culture of ATDC5 cells, cartilaginous nodules were formed through cellular condensation. Then, these cells exhibited the phenotype of chondrocyte, that is, secretion of type II collagen, aggrecan and other ECM molecules (early-phase differentiation). Hypertrophic chondrocytes appeared subsequently associating with elevation of type X collagen expression followed by the process of matrix mineralization (late-phase differentiation; Fig. 1).

ATDC5 cells could easily proliferate so that we could obtain vast amount of cells to set up in vitro culture system, which could mimic the cellular condensation during chondrogenesis in vivo. In addition, these cells could maintain the undifferentiated state during expansion. Thus, the well characterized chondrogenic cell line—ATDC5 is an excellent model to investigate molecular mechanisms of chondrogenesis in vitro. So far, ATDC5 cells have been widely used to explore the underlying mechanism of chondrocytic differentiation, including the signal pathways during endochondral bone formation, the roles of various exogenous biomolecules on chondrogenesis, changes in gene expression, etc. Also, this cell line has been adopted to assess the function of new-designed materials [Fujii et al., 1999; James et al., 2005; Mendes et al., 2012; Yamaura et al., 2012]. Next, we will discuss the role ATDC5 cells can play in the above uses.

IN VITRO STUDIES FOR REGULATION OF CHONDROGENESIS

Chondrogenesis is affected by a variety of factors including various cytokines, hormones, and transcriptional factors. The interaction between these factors as well as the important roles of many transcriptional factors in different phases during the chondrogenic differentiation have been reported by many in vitro studies using ATDC5 cells. Next, we discuss the research progress according to the following categories: (1) growth factors, (2) hormones, (3) transcriptional factors, and (4) other factors.

GROWTH FACTORS

Most studies focus on transforming growth factor-beta (TGF-β), which is a kind of multifunctional peptide growth factor. Han and the collaborators tried to examine the effect of TGF-β1 on chondrogenesis of ATDC5 cells without insulin treatment and investigate the relationship between TGF-β1 modulated fibronectin (FN) expression and chondrogenesis. The data suggested that TGF-β1 treatment promoted the early-phase chondrocytic differentiation and influenced FN isoform expression as well [Han et al., 2005]. Later, they demonstrated that TGF-β1 could modulate the splicing factor SRp40 isoform expression, thus leading to the regulation of FN splicing [Han et al., 2007]. Another group further explored the regulation of FN by TGF-β1. They mentioned that it was epidermal growth factor receptor activated by TGF-β1 that induced the expression of extracellular matrix gene FN [Takeda et al., 2010]. Following that, FN would liaise with integrins by RGDS peptide to mediate the balance between aggrecan and versican during chondrogenesis [Kutsuna et al., 2011]. Moreover, the influx of phosphate and calcium into ATDC5 cells was necessary for stimulation of ANK regulated by TGF-β1 [Oca et al., 2010]. Another member of TGF-β family, activin-A, has been indicated to suppress chondrogenesis of ATDC5 cells by evidence of lower expression of sox9 and runx2 genes as well as less synthesis of proteoglycan. The data suggested that the results were caused by inhibition of JNK and Akt pathways by activin-A [Mitsugi et al., 2012].

Bone morphogenetic proteins (BMPs) belong to TGF-β superfamily and they have the capability to stimulate the formation of bone and cartilage. During the process of chondrogenesis in ATDC5 cells, at least four members of BMPs were endogenously expressed [Akiyama et al., 2000]. BMP-4 was expressed throughout the chondrogenesis, whereas GDF-5 and BMP-6 were induced sequentially during the cellular condensation and the formation of cartilaginous nodules. And then BMP-7 showed up at the late-phase differentiation. Shukunami demonstrated that autocrine BMP-4 signaling was needed for induction of ATDC5 cells into chondrocytes [Shukunami et al., 2000]. The in vivo studies in chick limbs, consistent with the results from studies using ATDC5 cells, demonstrated that continuous BMP signaling was required in

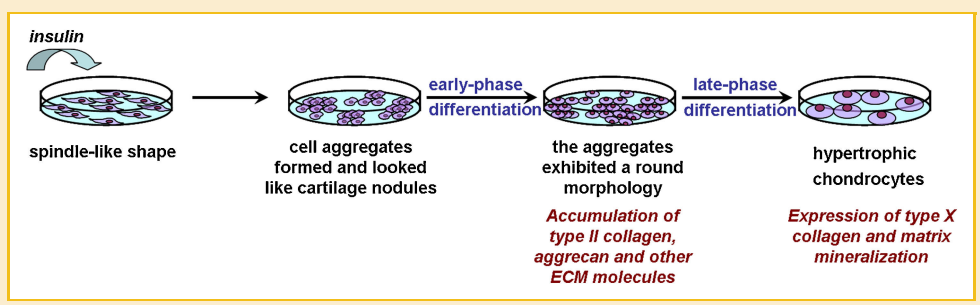

Fig. 1. Schematic diagram for transition of phenotype of ATDC5 cells during chondrogenesis.

chondrogenesis [Pizette and Niswander, 2000; Miljkovic et al., 2008]. It was further confirmed that splicing factor 3b subunit 4 could interact with type IA BMP receptor and specifically suppressed osteochondral cell differentiation regulated by BMP [Watanabe et al., 2007]. By means of serial analysis of gene expression (SAGE), over 100 transcripts were found to differentially expressed in ATDC5 cells before and after treatment of BMP-4 [Wahl et al., 2004]. Thereinto, 12 potentially novel genes were found while many of the known genes have never been implicated in chondrogenesis. All these findings have shed light on molecular events modulated by BMP signaling during chondrogenic differentiation. Besides, Fukui et al. found that the expression of BMP-2 in differentiated cells was stimulated by TNF-alpha via p38 signal pathway and augmented through transcriptional up-regulation via nuclear factor-kappaB (NF-κB) [Fukui et al., 2006]. BMP-2 also effectively promoted the expression of receptor activator of nuclear factor-kappaB ligand (RANKL) through regulation of RANKL promoter activity that was mediated by Runt-related transcription factor 2 (Runx2). By this means, hypertrophic chondrocytes may eliminate calcified matrix and thus modulate osteoclastogenesis [Usui et al., 2008]. BMP-5 was shown to enhance the expression of hypertrophy markers in ATDC5 cells by activating multiple signaling pathways including p38 MAPK, Smad, and extracellular signal-regulated kinase signaling [Snelling et al., 2010]. Some studies depicted that GDF-5 induced ATDC5 cells chondrogenesis was associated with Smad pathway [Sammar et al., 2004] and GDF-5 signaling was exclusively controlled via type IB BMP receptor [Kotzsch et al., 2009].

Recently, an interesting in vitro study using ATDC5 cells has brought new insight to the relationship between BMP and TGF-β signaling in endochondral ossification [Keller et al., 2011]. The researchers found that TGF-β1 remarkably enhanced BMP signaling while BMP-2 significantly inhibited TGF-β signaling through the use of luciferase reporter system. However, the mechanism of this BMP/TGF-β feedback loop remains to be further studied.

HORMONES

Parathyroid hormone-related protein (PTHrP), a protein member of the parathyroid hormone family, has been demonstrated as a key regulator in proliferation and hypertrophy of ATDC5 cells during chondrocytic differentiation. Shukunami et al. [1996] reported that exogenous PTHrP activated the receptor and then stimulated a series of complex cascades, which was in association with cellular condensation. The data from Ueda et al. [2007] suggested that PTH was involved in the inhibition of both differentiation and apoptosis of cells. The work by another group showed that PTHrP mediated the conversion of ATDC5 cells into hypertrophic chondrocytes. They found that PTHrP-(1-141) isoform downregulated the expression of type X collagen, a marker of hypertrophic chondrocytes while BMP-4 did conversely [Ito et al., 1999]. This group then demonstrated that PTHrP-(1-141) isoform suppressed the expression of BMP-4 mRNA through a cAMP/PKA pathway [Ito et al., 2000]. Some studies [Hoogendam et al., 2006, 2007] were focused on the identification of PTHrP target genes and uncovered the interaction between PTHrP and Janus kinase/Stat signaling.

Researchers observed that leptin, one of the adipose derived hormones, helped ATDC5 cells avoid apoptosis and regulated the events regarding the late-phase differentiation of chondrocytes [Kishida et al., 2005]. When leptin bound to its receptors, it would immediately trigger several complex signaling pathways associated with chondrogenic differentiation, including the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and mitogen-activated protein kinase (MAPK) pathways [Ben-Eliezer et al., 2007] as well as signaling pathway involving phosphatidylinositol 3-kinase (PI-3K), mitogen-activated protein kinase (MEK1), and p38 kinase [Otero et al., 2003, 2005, 2007]. In addition to downstream signaling through its receptors, leptin could also take part in crosstalk with estrogen receptors which in turn affected on the regulation of chondrogenesis via ERK signaling pathway [Wang et al., 2011, 2012].

Other hormones such as adiponectin [Challa et al., 2010], thyroid hormone [Miura et al., 2002], prolactin [Seriwatanachai et al., 2012], and adrenaline [Takarada et al., 2009] have also been studied. Adiponectin was proved to enhance the proliferation and differentiation of ADTC5 cells by upregulation of chondrogenic signaling molecules including Ihh, PTHrP, Ptc1, FGF18, BMP7, IGF1, and p21 [Challa et al., 2010]. Additionally, it was demonstrated as a key factor involved in maintaining the homeostasis of cartilage [Lago et al., 2008]. In contrast, thyroid hormone was shown to inhibit cell proliferation but increase ALP activity and facilitate matrix secretion. The data also suggested its function in regulating matrix-mediated paracrine signaling during endochondral ossification [Miura et al., 2002; Siebler et al., 2002].

TRANSCRIPTION FACTORS

Transcription factor play a critical role in controlling the flow of chondrogenesis from progenitor cells to chondrocytes and to the terminally differentiated hypertrophic chondrocytes. One of the most important transcription factors is Sox 9, which is essential for chondrocyte differentiation. Studies have been reported that the expression of Sox9 in ATDC5 cells was upregulated by a 30-bp element in the first intron of Sox9 [Morishita et al., 2001] and was induced by RelA, a NF-κB member, via specific binding in the Sox9 promoter [Ushita et al., 2009]. The transient activation of NF-κB/p65 stimulated the expression of Sox9 at the early phase of differentiation while inhibition of it led to the decrease of Sox9 [Caron et al., 2012]. Saito et al. [2007] identified S100A1 and S100B as transcriptional targets of Sox trio (Sox9 and its coactivators Sox5, Sox6) which would modulate the induction of early-stage chondrocyte differentiation and inhibition of the late-stage. Besides, a series of work by Yoneda group [Hata et al., 2008; Takigawa et al., 2010; Amano et al., 2011] focused on exploring the molecular mechanisms of Sox9 for chondrogenesis. By screening the cDNA library of the ATDC5 cell line, they found that paraspeckle protein p54nrb, Znf219, and Arid5a would interact with Sox9 and play a role in promoting chondrocyte differentiation.

Runx2, also known as core-binding factor subunit alpha-1 (CBF-alpha-1), is a vital transcription factor involved in osteoblast and chondrocyte differentiation. Forced expression of Runx2 not only stimulated cell migration, but also promoted chondrogenic differentiation of ATDC5 cells through phosphatidylinositol 3-kinase (PI3K)-Akt signaling [Fujita et al., 2004a]. Runx2 also led to

the accumulation of the hypoxia-inducible factor-1alpha in ATDC5 cells by binding to oxygen-dependent degradation domain, thus inducing angiogenesis during endochondral ossification [Lee et al., 2012]. Another Runx family member, Runx1, was found as a downstream target of Trps1 by a DNA array. Trps1 was demonstrated to control the proliferation and apoptosis of ATDC5 cells via Stat3 signaling [Suemoto et al., 2007]. The binding of Trps1 to the P2 promoter in Runx1 gene facilitated normal cartilage formation [Kanno et al., 2011].

In addition, some studies identified more transcription factors associated with endochondral ossification. Nuclear factor I-B was found to induce early-stage of chondrogenic differentiation [Uchihashi et al., 2007] while basic helix-loop-helix transcription factor DEC1 was shown to affect both early-stage and terminal differentiation [Shen et al., 2002]. In contrast, transcription factor nuclear factor E2 p45-related factor 2 (Nrf2) showed negative influence on chondrogenesis [Hinoi et al., 2007]. However, further studies are needed to uncover the underlying mechanisms.

OTHER FACTORS

Lots of efforts have been exerted on the role of inorganic phosphate (Pi) and its related mechanism in endochondral ossification by use of ATDC5 cells. Guicheux et al. established a promising in vitro model system with the ATDC5 cell line to analyze the function of Pi during the process of endochondral ossification [Guicheux et al., 2000]. Since then, a lot of research work has been carried out. The data suggested that Pi contributed to regulation of chondrocytes maturation and mineralization involved with apoptosis, evidenced by increased expression of type X collagen, calcium content as well as augmented DNA fragmentation and caspase-3 activity [Magne et al., 2003; Denison et al., 2009]. Pi was reported to induce nuclear export of Runx2 to speed up the mineralization [Fujita et al., 2001]. The researchers demonstrated that Pi caused activation of ERK1/2 signaling pathway, which was involved in modulating an inhibitor of mineralization-matrix Gla protein [Julien et al., 2007]. This was consistent with another study [Kimata et al., 2010], which suggested that Pi activated the Raf/MEK/ERK pathway through mediation by Pit-1 and FRS2alpha.

Dexamethasone and ascorbate are two main exogenous elements with effect on skeletal development. Although dexamethasone was shown to inhibit growth of ATDC5 cells and matrix formation [Mushtaq et al., 2002], the chondrogenic program of ATDC5 cells could be reset once dexamethasone was withdrawn [Siebler et al., 2002]. With the treatment of dexamethasone in ATDC5 cells, PI3K-Akt signaling was inhibited as well as Runx2-dependent transcription was suppressed in dose-dependent manner [Fujita et al., 2004b]. Another lab found that lipocalin 2 was involved in the regulation of dexamethasone effects on chondrocytes by means of Affymetrix microarray analysis of ATDC5 cells [Owen et al., 2008]. On the contrary, ascorbate exhibited the enhanced effect on chondrogenesis of ATDC5 cells [Altaf et al., 2006]. Ascorbate was beneficial for collagenous matrix production, which in turn activated ERK pathway and accordingly promoted the differentiation [Temu et al., 2010].

APPLICATION IN EVALUATION OF NEW MATERIALS

Aside from the above factors, 3D environment is of great importance for chondrogenesis. To this end, numerous creative materials come forth. It is necessary to set up a convenient and stable model system to evaluate the function of these materials. Due to its extensive and rapid proliferation along with homogeneity as well as chondrogenic capacity, the ATDC5 cell line is regarded as a promising model cell line for chondrogenesis. Thereby, more and more researchers have adopted ATDC5 cells to evaluate the effects of new materials on cell behaviors, viability, growth, and chondrogenesis [Silva et al., 2008; Tigli and Gumusderelioglu, 2009; Malafaya et al., 2010; Miranda et al., 2011; Sa-Lima et al., 2011; Yang et al., 2011; Yunos et al., 2011; Mendes et al., 2012].

In addition, this model cell line could be used to obtain some tips so as to guide the design of materials as well as optimize the parameters. Kwon et al. [2010] made a comparison among synthetic polymer gels with various charge densities to investigate the influence on chondrogenesis of ATDC5 cells. The results suggested that highly negatively charged gel could promote the chondrogenic differentiation of ATDC5 cells even without the addition of insulin, indicating that negative charge was favorable for chondrogenesis. Thus, this element should be considered for the future design of materials.

A recent study compared various kinds of chondrogenic differentiation media used in previous studies of ATDC5 cells and demonstrated that the ATDC5 cells favored the medium with supplement of ascorbic acid, dexamethasone, ITS, and TGF-β1 for chondrocytic differentiation [Weiss et al., 2012]. Therefore, the model system using ATDC5 cells should be continuously developed so that the ATDC5 cell line could play a better role as a model. Through the setup of putative standard for this model system, the results could be more consistent and reliable, and thus evaluation of materials could be done more effectively and efficiently.

SUMMARY AND FUTURE DIRECTION

Endochondral ossification is a complex series of events, in which the mechanisms of various cytokines, hormones, and so on have not been clearly elucidated. To uncover the truth, a stable and recognized model system is required. The ATDC5 cell line appears as an excellent in vitro cell model evident by a number of studies. ATDC5 cells are homogeneous and can be kept undifferentiated under normal culture conditions with rapid growth rate. Although these cells cannot replace mesenchymal stem cells, they can provide important information about optimal parameters such as seeding densities and dosage of growth factors, for tissue engineering strategies, thereby significantly saving time and cost as compared to mesenchymal stem cells that are easily influenced by the source and passage number. Meanwhile, these in vitro studies and models may provide fundamental biological insights as key references for future animal/clinical trials.

Researchers also exerted themselves to examine the effects of chondrogenesis in ADTC5 cells by other conditions, such as oxygen

conditions (different oxygen concentrations) [Chen et al., 2006; Zaka et al., 2009], laser irradiation [Kushibiki et al., 2010], and mechanical interaction [Xing et al., 2011; Kambe et al., 2012]. Lots of findings have been obtained and could potentially provide the clues for regenerative medicine for damaged cartilage. Furthermore, recent studies have identified several new therapeutic targets for various skeletal diseases via ATDC5 cell model [Hojo et al., 2010; Tsuritani et al., 2010; Chen et al., 2011; Choi et al., 2011; Koshimizu et al., 2012]. However, their mechanisms have not been fully understood. By means of in vitro model system, future research should focus on the understanding of molecular events which regulate chondrogenesis as well as the mechanisms involved in the endochondral ossification and congenital skeletal deformities. Consequently, this helps us find out potential candidates and prepare preferable biomaterials for bone/cartilage repair and tissue engineering strategies.

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