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ICAT participates in proliferation and osteogenic differentiation of human adipose tissue-derived mesenchymal stem cell

Yeon Jeong Kim [a,b], Jong Tae Kim [a,b], Yong Chan Bae [c], Kuen Taek Suh [d,*], Jin Sup Jung [a,b,e,*]

[a] Department of Physiology, School of Medicine, Pusan National University, Pusan, Republic of Korea
[b] Medical Research Center for Ischemic Tissue Engineering, Pusan National University, Pusan, Republic of Korea
[c] Department of Plastic Surgery, School of Medicine, Pusan National University, Pusan, Republic of Korea
[d] Department of Orthopedic Surgery, School of Medicine, Pusan National University, Pusan, Republic of Korea
[e] Medical Research Institute, Pusan National University, Pusan, Republic of Korea

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ABSTRACT

Aims: The Wnt/β-catenin pathway plays a critical part in several cell physiology events associated with embryonic development and adult homeostasis, including determination, proliferation, migration, and differentiation. However, the role of Wnt signaling in osteoblastogenesis from mesenchymal stem cells (MSC) remains a controversial matter. Therefore, in the present study, we investigated how ICAT (inhibitor of β-catenin and TCF-4), a negative regulator of the Wnt signaling pathway, influenced differentiation and proliferation of human adipose tissue-derived stromal cells (hASC).
Main methods: To mediate ICAT overexpression in hASC, we used a lentiviral gene transfer technique. We further determined the role of ICAT by RNAi technique.
Key findings: ICAT-transduced hASC exhibited lower TCF promoter activity and cellular growth capacity than control cells, but ICAT overexpression did not affect hASC attachment efficiency. ICAT overexpression also increased osteogenic differentiation. Conversely, introduction of an ICAT siRNA oligonucleotide increased TCF promoter activity and cellular proliferation, but it inhibited osteogenic differentiation.
Significance: Taken together, these findings indicated that ICAT participated in regulating hASC proliferation and differentiation by modulating Wnt signaling.

Introduction

β-catenin is a cytoplasmic protein with dual functions in cell–cell adhesion and signal transduction. At the plasma membrane, β-catenin associates with adhesion molecules called cadherins, linking them to the actin cytoskeleton and ensuring strong cellular adhesion. In contrast, soluble β-catenin is a downstream component of the Wnt signal transduction pathway, which is involved in many aspects of embryonic patterning; for example, dorsal–ventral axis determination in *Xenopus*.

Unstimulated cells keep this soluble pool of β-catenin at extremely low levels, using constitutive phosphorylation, ubiquitination, and degradation. Activation of the Wnt pathway inhibits the phosphorylation/ubiquitination machinery and creates a large pool of soluble unphosphorylated β-catenin that is free to enter the nucleus. In the nucleus, β-catenin interacts with the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and activates transcription of downstream genes such as c-Myc, cyclin D1, and Axin2 (He et al., 1998; Tetsu and McCormick, 1999; Yan et al., 2001; Leung et al., 2002; Lustig et al., 2002; Stow, 2004).

ICAT (inhibitor of β-catenin and TCF-4) is an 81-amino acid protein that competitively inhibits β-catenin/TCF signaling in both reporter gene and *Xenopus* axis formation assays. Humans, mice, rats, zebrafish, and frogs all possess highly-conserved orthologs of ICAT that have no homology to other known proteins (Gottardi and Gumbiner, 2004; Tago et al., 2000).

Like bone marrow, adipose tissue is a mesodermally-derived organ that contains a stromal population among microvascular endothelial, smooth muscle, and stem cells (Zuk et al., 2001; Lee et al., 2004; Kim et al., 2007). The stem cells can be enzymatically digested out of adipose tissue and separated from the more buoyant adipocytes by centrifugation. This population, termed adipose tissue-derived mesenchymal stem cells (ASC), shares many of the characteristics of its counterpart population in bone marrow, including extensive proliferative potential and the ability to form adipogenic, osteogenic, chondrogenic, and myogenic lineages (Zuk et al., 2002; Rodriguez et al., 2005; Gimble and Guilak, 2003). Previous studies (Zuk et al., 2002; Rodriguez et al., 2005; Gimble and Guilak, 2003; Hicok et al., 2004; Kim et al., 2006) have described the osteogenic potential of hASC,

* Corresponding authors. Jung is to be contacted at Department of Physiology, School of Medicine, Pusan National University, 1 Ga, Ami-Dong, Suh-Gu, Pusan 602-739, Republic of Korea. Tel.: +82 51 240 7734; fax: +82 51 246 6001. Suh, Department of Orthopedic Surgery, School of Medicine, Pusan National University, 1 Ga, Ami-Dong, Suh-Gu, Pusan 602-739, Republic of Korea. Tel.: +82 51 240 7248; fax: +82 51 246 6001.
E-mail addresses: kuentak@pusan.ac.kr (K.T. Suh), jsjung@pusan.ac.kr (J.S. Jung).

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demonstrating their in vivo bone-forming capacity, but the molecular mechanisms underlying hASC differentiation toward the osteoblastic phenotype are still unknown.

Recent studies implicated Wnt signaling in proliferation and differentiation of MSC (Boland et al., 2004; Cho et al., 2006). In humans, loss-of-function mutations in a Wnt co-receptor, the low-density lipoprotein receptor-related protein 5 (LRP5), result in osteoporosis–pseudoglioma syndrome, a disorder characterized by low bone mass and skeletal fragility (Gong et al., 2001). In contrast, LRP5 gain-of-function mutations yield a high bone mass phenotype (Boyden et al., 2002; Little et al., 2002). Activation of Wnt signaling has been reported to increase osteogenic differentiation of murine mesenchymal cell lines (Rawadi et al., 2003; Hu et al., 2005). However, it has been reported that the addition of Wnt3a or the treatment of lithium inhibits osteogenic differentiation of naïve human MSC derived from bone marrow (Boland Et al., 2004; de Boer et al., 2004) and adipose tissues (Cho et al., 2006). Overexpression of lymphocyte enhancer-binding factor 1 (lef-1), a transcriptional regulator of the Wnt/beta-catenin signaling cascade, inhibited terminal differentiation of osteoblasts (Kahler et al., 2006), and the downregulation of Wnt signaling was required for the late-stage differentiation of osteoblasts (van der Horst et al., 2005).

To unravel the controversy regarding the role of Wnt signaling in osteogenesis, herein we investigated how the negative regulator ICAT affects the differentiation and proliferation of hASC.

Materials and methods

Cell culture

All protocols involving human subjects were approved by the Institutional Review Board of the Pusan National University. Adipose tissues were obtained from individuals undergoing elective abdominoplasty after obtaining informed consent from each individual. The patients were 55 years old male, 36 years old male, 43 years old female and 29 years old female. The hASC were isolated from adipose tissues according to the methods described in the previous studies. Briefly, adipose tissues were digested at 37 °C for 30 min with 0.075% type I collagenase (Lee et al., 2004). The enzyme activity was neutralized with α-modified Eagle's medium (α-MEM), containing 10% fetal bovine serum (FBS), and the mixture was centrifuged at 1200 ×g for 10 min to obtain a pellet. The pellet was incubated overnight at 37 °C/5% CO_2 in a control medium (α-MEM, 10% FBS, 100 units/ml of penicillin, 100 μg/ml of streptomycin). Following incubation, the tissue culture plates were washed to remove any residual nonadherent cells and were maintained at 37 °C/5% CO_2 in the control medium. When the monolayer of adherent cells reached a level of confluence, the cells were trypsinized (0.25% trypsin; Sigma, USA), resuspended in α-MEM containing 10% FBS, and subcultured at a concentration of 2000 cells/cm^2. We used the 3rd–5th passages of hASC for this experiment.

Lentiviral vector construction and transduction

The cDNA for human ICAT was generated with RNA from hASC by using the RT–PCR method (5′-CACCGAAGCAGGAGTCCCCAGAG-3′, 5′-AGGGTGTTCCAAGGGCTTT-3′). The 4 base pair sequences (CACC) was included necessary for directional TOPO® cloning on the 5′ end of the forward primer. The human ICAT gene was cloning by using the pENTR™/SD/D-TOPO® Cloning Kit (Invitrogen, CA), according to the manufacturer's specified guidelines. The ICAT gene of pENTR™/SD/D-ICAT was recombined into pLenti6/V5 using LR clonase (Invitrogen, CA) resulting a pLenti6/V5-ICAT plasmid. The plasmids were sequenced with the primer to confirm the correctness of constructed plasmid. Replication-defective lentiviruses were produced by transient transfection of 293T cells using Lipofectamine Plus (Invitrogen, CA), lentivirus vectors, and packaging mix (Invitrogen, CA). After transfection, two samples of viruses were harvested at 48 and 72 h, and filtered through Millex-HV 0.45 μm PVDF filter (Millipore, MA) and kept at −80 °C until use. Transduction of hASC was performed by 6 h of exposure to dilutions of viral supernatant in the presence of Polybrene (5 μg/ml). The transductants were selected by culture in blasticidin (10 ng/ml; Invitrogen, CA).

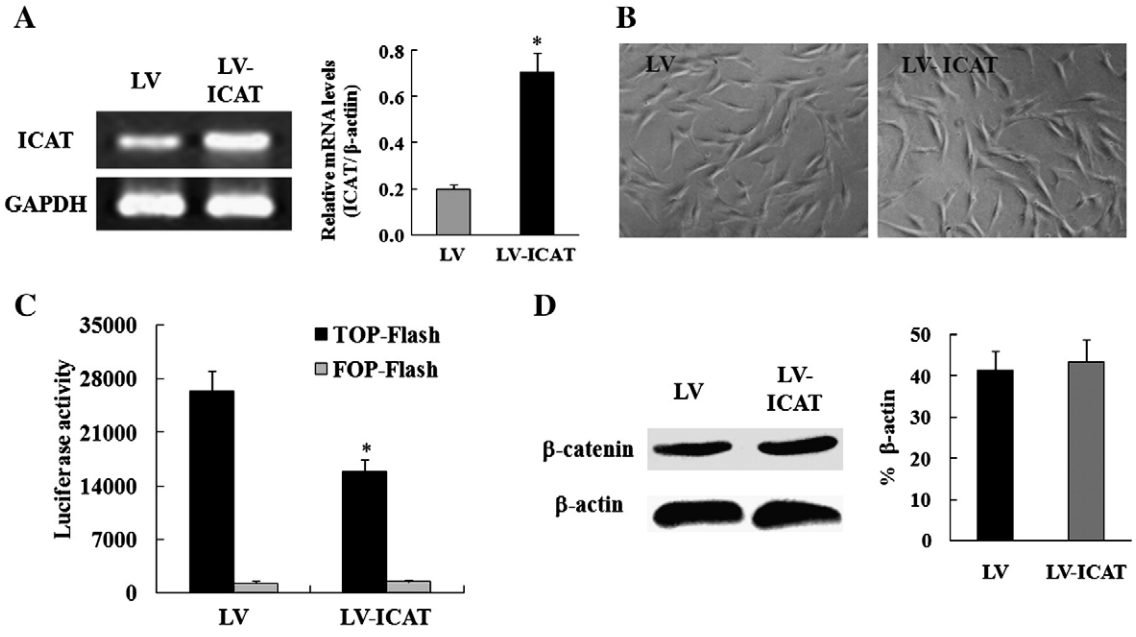

Fig. 1. Effect of β-catenin–TCF-4-mediated transcription in ICAT-transduced hASC. (A) ICAT mRNA levels were determined by RT–PCR of pLenti-vector (LV) or pLenti-ICAT (LV-ICAT) transduced hASC. Quantitation was performed by real time PCR analysis. Data represent mean ± SEM of the relative ratio to β-actin signal of the corresponding samples (n=4). (B) ICAT expression levels did not affect cell morphology, as shown in representative phase contrast images (200×). (C) Lentivirus-transduced hASC were transfected with pTOP-Flash (containing triple TCF/LEF1 binding sites, a basic thymidine kinase promoter, and a *firefly* luciferase reporter gene), pFOP-Flash (containing mutated TCF/LEF1 binding sites), or pCMV-β-Gal constructs. Luciferase activity was measured two days after transfection and normalized to β-galactosidase activity. (D) β-catenin expression in LV or LV-ICAT hASC was analyzed by western blot, using β-actin expression as a loading control. β-catenin expression levels were quantified with an image analysis program. Data represent mean ± SEM (n=4). *P<0.05 compared with LV-transduced-hASC.