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Pro-fibrotic effects of PFKFB4-mediated glycolytic reprogramming in fibrous dysplasia

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ABSTRACT

Fibrous dysplasia (FD) caused by a mosaic somatic mutation of *GNAS* is characterized by replacement of the affected bone with abnormal fibrous tissue. Herein, we present novel disease models for FD developed with pairs of isogenic wild-type and $GNAS^{R201H}$-mutated induced pluripotent stem cells (iPSCs) and their derivative mesenchymal stem cells (MSCs). Both 2D and 3D MSC culture models for FD successfully reflect FD's typical molecular characteristics, such as enhanced cAMP level, PKA activity, CREB1 phosphorylation and the pathologic fibrotic phenotype. The fibrotic features of $GNAS^{R201H}$ FD model cells were closely linked to augmented glycolysis and depended on glycolytic PFKFB4 and the activation of pro-fibrotic TGFβ signalling. Either depletion of PFKFB4 or inhibition of glycolysis or TGFβ signalling potentially blocked fibrosis progression in $GNAS^{R201H}$ FD model cells. Our FD models could facilitate a better mechanistic understanding of FD and help develop effective therapeutics for FD and other fibrosis diseases.

1. Introduction

Fibrous dysplasia of bone (FD; a benign fibro-osseous bone dysplasia) is a rare, non-inheritable skeletal disorder that can affect any part of the bone. FD disturbs normal bone architecture, structure, and mineral content and results in severe pain, fractures/deformities [1–3]. The treatment options are mainly palliative, and medicines are used for symptomatic relief but are not capable of altering the disease progression. Thus, the study and management of human FD remains a constant challenge.

At the molecular level, the cause of FD is somatic post-zygotic missense mutations of codon 201 in exon 8 of the *GNAS* (Gsα) gene that lead to approximately 30-fold reduced intrinsic GTPase activity, constitutive elevation of cyclic adenosine monophosphate (cAMP) and an overactive cAMP-dependent signalling pathway [4,5]. The involvement of the CREB-Smad6-Runx2 axis in FD osteogenesis dysfunction was revealed by investigating of human FD patient's bone marrow stromal cells (FD-BMSCs) carrying a $GNAS^{R201H}$ mutation and excess cAMP-treated BMSCs [6]. Proper modulation of Gsα-Wnt/β-catenin signalling is required for normal bone formation; however, defective activation of Gsα resulted in elevated Wnt/β-catenin signalling in FD-BMSCs [7]. The detection of excess production of *IL-6* [8] and up-regulation of *RANK-L* [9] causing bone resorption in FD BMSCs implied their involvement in the increased osteoclastogenesis and bone loss observed in FD lesions. Likewise, pathogenic effects of the *GNAS* mutation in human FD have been mainly revealed via *in vitro* assays with mutated skeletal stem/progenitor cells isolated from FD bone lesions [4,5]; however, research models with the defining features of human FD, such as marrow fibrosis, are lacking. An *in vivo* transplantation assay with mutated skeletal cells [10] is the only method to recapitulate the fibrous phenotype of human FD; thus, it is difficult to study the disease mechanism and therapeutics. Accordingly, the fundamental mechanism of fibrotic marrow in FD remains

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Metabolic perturbations are often related to disease outbreaks [11]. In particular, cancer is a prime example of a human disease with pathological metabolism that contributes to tumour initiation, progression and malignant transformation [12]. Enhanced glycolysis and impaired oxidative phosphorylation are hallmarks of cancer, and most cancers outcompete the surrounding tissues in glucose uptake [12]. Thus, these metabolic characteristics have allowed the development of a diagnostic imaging method for cancer using ^{18}fluroro-2-deoxyglucose positron emission tomography (FDG-PET). Although abnormal metabolism has not been reported in FD pathogenesis, interestingly, several clinical reports of FDG-PET from FD patients showed enhanced glucose uptake in the fibrotic lesion compared to normal surrounding tissues [13–15]. These clinical observations imply the possible involvement of glucose metabolism in FD pathogenesis; however, the role of glucose metabolism in fibrous tissue development by GNAS mutation is completely unknown.

Currently, patient-derived human induced pluripotent stem cells (hiPSCs) generated by forced expression of key reprogramming factors provide a valuable source of patient- and disease-specific cell types. In this study, we developed a novel FD bone fibrosis model with 2D or 3D culture using mesenchymal stem cells (MSCs) differentiated from FD patient-derived hiPSCs. Based on our FD *in vitro* model, we found that PFKFB4-induced glycolytic reprogramming is important for pro-fibrotic responses by creating an acidic microenvironment and sequentially activating the transforming growth factor β (TGFβ) pathway in human FD. We also suggest that glycolytic inhibition could be a valuable therapeutic approach for FD patients and other fibrotic disorders.

2. Materials and Methods

2.1. Patient samples

For fibroblasts isolation, skin and/or fibrotic bone tissues were obtained from biopsies of five female FD patients. FD skin fibroblasts had no GNAS mutation while GNAS mutations (R201H or R201C) were cells from FD fibrotic bone tissues. An isogenic pair of FD skin and bone fibroblasts were used for iPS generation. This study was approved by the Public Institutional Review Board (Seoul, Korea; IRB no. P01-201404-BS-05).

2.2. Reprogramming, differentiation and bone spheroid culture

The generation and characterization of induced pluripotent stem cells were performed as previously described [16]. To differentiate iPSCs into MSCs, FD-iPSs were transferred to Matrigel (Corning, Cat no. 354277)-coated dishes with TESR-E8 medium (Stemcell Technologies, Cat no. 05940) and cultured for 5 days. Then, FD-iPSs were differentiated by changing the medium to alpha-MEM (Gibco, Cat no. 12571) supplemented with 10% fetal bovine serum (FBS; Gibco, Cat no. 16000) and 5 ng/ml basic fibroblast growth factor (bFGF) for one month. Differentiated MSCs were passaged every 3 days. The characterization of iPSs or MSCs was performed as previously described [17], and detailed protocol was described in the supplementary methods.

Bone sphere culture was performed as previously described [18]. To stain the bone sphere, spheroids were fixed with 4% paraformaldehyde (PFA) and embedded in Tissue-Tek OCT (Optimal Cutting Temperature) Compound (Sakura Finetek USA, Inc., Cat no. 4583) for frozen sectioning.

2.3. ELISA (measurement of cyclic AMP and active TGFβ)

To measure cAMP levels, we performed AChE (Acetylcholinesterase) Competitive ELISAs (cyclic AMP EIA Kit, Cayman Chemical, Cat no. 581001) following the manufacturer's protocols. For the active TGFβ assay, conditioned medium was replaced with low-serum medium (0.1%) for 24 h, and then active TGFβ was measured using a Human TGF-β1 Quantikine ELISA Kit (R&D Systems, Cat no. DB100B) following the manufacturer's protocols.

2.4. Glycolysis assay

To examine the glucose utilization rate, the levels of residual glucose in the cultured medium were determined using a Glucose (GO) assay kit (Sigma-Aldrich, Cat no. GAGO-20). The amount of glucose consumed in 24 h was calculated by subtracting the amount of residual glucose from the initial amount of glucose in the medium. Furthermore, the levels of lactate (a final product of glycolysis) were measured using a Lactate assay kit 2 (Biovision, Cat no. K627-100) following the manufacturer's protocols.

2.5. Fibrillary collagen staining

To stain extracellular collagen, we used Picrosirius Red and Masson's trichrome staining. Each staining was performed using a staining kit from Polysciences, Inc. (Picrosirius Red Stain Kit, Cat no. 24901; Masson's Trichrome Stain Kit, Cat no. 25088-1).

2.6. Transcriptome analysis

To compare global transcription patterns, MSCs were starved in low-serum medium (1% FBS) for 24 h, and then TGFβ (5 ng/ml) was treated for 24 h. Total RNA was extracted with an easy-BLUE total RNA extraction kit (Intron Biotechnology, Cat no. 17061), and a cDNA microarray was performed using an Agilent Human GE (V2) 4 × 44K chip. Data of each sample were normalized to wt-MSC-1 using Agilent's GeneSpring software, and then statistically significant differentially expressed genes (DEGs) were analysed using MultiExperiment Viewer software (MeV version 4.9.0; available at: http://www.tm4.org) [19]. Hierarchal clustering and distance matrix analysis were also performed using MeV. For bioinformatics analysis, we used several plugins from Cytoscape software (version 3.1.0. available at: www.cytoscape.org) [20]. Specifically, the Reactome FI Plugin was used for analysis of the functional interaction (FI) network with linker proteins and pathway enrichment, the MCODE Plugin was used to find highly interconnected pathways. In addition, the PANTHER classification system was used to analyse the biological processes of significant disease genes (http://pantherdb.org/).

2.7. Metabolite analysis

To analyse metabolite levels, MSCs were starved with low-serum medium (1% FBS) for 24 h, and then TGFβ (5 ng/ml) was treated for 24 h. Cells were washed with 5% (w/w) mannitol solution, and metabolite was extracted by methanol. Residual cell debris or proteins were removed by ultrafiltration, and then the metabolites were evaporated. The metabolite analysis was performed by capillary electrophoresis–time-of-flight/mass spectrometry (CE-TOF/MS) analysis (C-SCOPE, Human Metabolome Technologies, Inc.). Statistically significant differential metabolites were analysed in MeV software using two-way ANOVA methods.