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Research paper

Epigenetic modifications and mRNA levels of the imprinted gene *Grb10* in serially passaged fibroblast cells

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ABSTRACT

The imprinted gene *Grb10* influences fetal growth, adult behavior and tumor formation. Investigating this gene often involves consecutive passaging of cells *in vitro*. We analyzed changes in *Grb10* DNA methylation and histone modifications in serially passaged adult mouse tail-tip fibroblast cells with bisulfite genomic sequencing method and chromatin immunoprecipitation (ChIP) respectively. There was a significant reduction in global DNA methylation, but no changes in methylation at CpG island 1 (CGI1) of *Grb10*. There were no changes in methylation at histone H3K4me2, but less methylated histone H3K27me3 was detected, along with more of the acetylated histones H3Ac and H4Ac. The major-type transcript level of *Grb10* in fibroblasts increased mildly after extended cultivation. These results suggest consecutive passaging may affect epigenetic modifications of *Grb10* in adult fibroblast cells.

1. Introduction

Grb10 encodes the Growth Factor Receptor Bound protein 10 (*Grb10*), a negative regulator in the signal transduction pathways for insulin and/or insulin-like growth factors (IGFs) [1]. It belongs to a small family of cytoplasmic adaptor proteins, which mediate the coupling of a number of cell surface receptor tyrosine kinases and signaling molecules with specific signal pathways [1]. It plays an important role in distinct physiological processes, including fetal growth, adult behavior and tumor formation [2–4].

Previous work has suggested the gene evolved to modulate social behavior [4]. Abnormal expression of *Grb10* leads to fetal growth retardation [5]. Furthermore, it was recently found that the adaptor protein GRB10 is an mTORC1 (mammalian target of rapamycin, mTOR) substrate that mediates the inhibition of phosphoinositide-3-kinase (PI3K). This is typical of cells lacking tuberous sclerosis complex, TSC (TSC2), a tumor suppressor and negative regulator of mTORC1, suggesting that *Grb10* might be a tumor suppressor protein regulated by mTORC1 [2,3,6].

Grb10 is a crucial imprinted gene, which is conserved in mice and humans [7,8]. *Grb10* has tissue-specific and promoter-specific expression, due to reciprocal imprinting, depending on the promoter. In most tissues, including the cultured tail-tip fibroblasts used in this study, *Grb10* is expressed exclusively from the major-type promoter in CGI1 (CpG island 1). In the brain, the brain-type transcript is expressed predominantly from the brain-type promoter [9].

It is often necessary, and inevitable, that fibroblasts undergo consecutive passaging *in vitro* during the research process. For example, to obtain transgenic and cloned large animals in somatic cell nuclear transfer (SCNT), the donor cells are often transfected fibroblasts that have been selected after consecutive passaging. Additionally, serially passaged fibroblasts are sometimes derived from an adult patient.

A further consideration is that a future application of SCNT is therapeutic cloning. If the object of therapeutic cloning is an adult patient, fibroblasts from the adult patient are considered to be the most convenient donor cells. It is also the case that consecutive passaging *in vitro* for adult fibroblasts is necessary for the production of induced pluripotent stem (iPS) cells during patient-specific therapy in adult patients [10].

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It has been reported that serial culture could change the epigenetic status of imprinted genes in embryonic stem cells [11]. Epigenetic processes involve chemical modifications to DNA or histones, which are closely associated with DNA and form the cores of chromatin packaging. Chromatin modifications are often termed epigenetic marks, and some of these are associated with transcription [12,13].

We suggest that similar modifications could occur in adult fibroblasts during serial culture. Due to the obvious importance of *Grb10*, and the fact abnormalities could affect research applications, we have chosen to analyze epigenetic modifications and changes in major-type transcript level of this imprinted gene in serially passaged adult fibroblasts.

2. Materials and methods

2.1. Establishment of fibroblast cells and culture maintenance

Experiments were approved by the Ethics Committee on Animal Experiments of Fujian Medical University. Primary cultures of tail-tip fibroblast cells were established from seven to eight week old mice (B6D2F1). The mouse tail-tip tissue was used for primary cultures, using the tissue piece cultivation method [14]. Cultures were passaged by releasing cells with trypsin, and re-seeded at an initial concentration of 100,000 cells/25 cm^2 flask. For long-term cultures, half of the culture medium (DMEM/F12 containing 10% fetal calf serum, Gibco) was changed every 3–4 days. Population doublings (PDs) were calculated at every passage using the following formula: log (final concentration/initial concentration) × 3.33 [15]. Tail-tip fibroblasts of early passage (PDs 2–3) and late passage (PDs 18–20) (marked EPD and LPD respectively) were collected for future analysis.

2.2. CpG and DNA methylation analysis in mouse Grb10

CpG islands are defined as being a 200-bp region of DNA with a high G–C content (greater than 50%) and an observed CpG:expected CpG ratio (ObsCpG:ExpCpG) of greater than or equal to 0.6. The sequence is from AL663087 (GenBank). Using the CpGPLOT software (http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html), CpG islands (CGIs) 1 and 2 were identified. A third weak CpG island (CGI3), located between CGI1 and CGI2, was identified in the mouse sequence using the Webgene CpG island prediction program (http://www.itb.cnr.it/sun/webgene/).

Isolated DNA was treated with sodium bisulfite using a CpG genome DNA modification kit (Chemicon International Inc, Temecula, CA), according to manufacturer protocol. Bisulfite-treated DNA samples were subjected to nested PCR amplification of CGI1. Initial PCR was carried out with the first primer set listed in Table 1, and the products diluted 100 times. These were then used as templates for nested PCR using the second primer set. The nested PCR products were cloned into the TA cloning vector (pMD18-T), and at least 10 clones for each sample were sequenced.

2.3. RNA extraction and cDNA synthesis

Total RNA was isolated from pooled cultured tail-tip fibroblasts of early and late passages using the Trizol reagent (BBI) according to manufacturer protocol. Purity of extracted RNA was measured at optical densities of 230, 260 and 280 nm, with a Nanodrop Photometer (Eppendorf, Hamburg, Germany). The RNA integrity was verified using ethidium bromide staining of 28S and 18S ribosomal RNA bands on 2% agarose gel. RNA was treated with amplification-grade DNaseI (Invitrogen) to degrade any genomic DNA present in the sample. cDNAs were generated from total RNA with ReverTra Ace-α-TM (TOYOBO) primed oligo (dt) 20 primers. For cDNA synthesis, 4 μl 5 × RT buffer, 2 μl dNTP mix (10 mM each dNTP), 1 μl Oligo-dT primer (10 μM), 1 μl (10 IU) RNase inhibitor and 1 μl reverse transcriptase were added. The first-strand cDNA was synthesized at 42 °C for 20 min. Then, mRNA–cDNA chains were denatured and reverse transcriptase activity was arrested by heating to 99 °C for 5 min. An identical reaction was carried out without reverse transcriptase as a negative control.

Table 1
Primer pairs used in real-time PCR and DNA methylation.

Function(s) and name	Primer sequence 5′–3′	Product size (bp)
Transcription analysis		
Grb10-Q-F	CACGAAGTTTCCGCGCA	315
Grb10-Q-R	AGTATCAGTATCAGACTGCATGTT	315
Gapdh-Q-F	TGTGTCCGTCGTGGATCTGA	150
Gapdh-Q-R	TTGCTGTTGAAGTCGCAGGAG	150
DNA methylation analysis		
Mel 1st-F	TGGGGTTTAATATTAAGTTTGA	365
Mel 1st-R	TTACATCTCTTAAATAAAACA	365
Mel 2nd-F	TGGGGTTTAATATTAAGTTTGA	271
Mel 2nd-R	AAATCACCTATAACTCTCCTAC	271
ChIP-QPCR analysis		
Q-ChIPF	CGTGTGAGTAGCCGAAGGAG	176
Q-ChIPR	CCGATCAAATTAGCCCGTT	176

2.4. Real-time RT-PCR

Quantitative analysis of each cell sample was carried out using quantitative real time PCR (qRT-PCR) of cDNA, with SYBR green and an ABI Prism 7500 (Applied Biosystems, California, USA). Two-step qRT-PCR of double-curve standard was performed on samples in triplicate, to control for PCR variations, and according to manufacturer protocol. Each experiment was performed three times on independent RNA extracts from three different mice. The quantity of each measured cDNA sample was normalized to the reference gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) [16,17].

Primers for amplification of *Grb10* major-type transcript have been previously described [18] and are listed in Table 1. Single product amplification was verified by solubility curve and electrophoresing qRT-PCR products on 1% agarose gels. The PCR efficiency for each single primer pair was determined using serial five-fold dilutions of cDNA transcripts. The linear correlation coefficient (R^2), an indicator of fit for the standard curve, which was plotted to the standard data points of all genes, ranged from 0.983 to 0.999. Cycling conditions were 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s, then 60 °C for 34 s.

2.5. Chromatin immunoprecipitation (ChIP)s

A ChIP assay was performed with a ChIP assay kit (Upstate Biotechnology) according to manufacturer protocol. Antibodies against acetylated histone H3 (H3Ac; catalog no. 06-599), acetylated histone H4 (H4Ac; catalog no. 06-866), Lys4 dimethylated histone H3 (H3K4me2; catalog no. 07-030), and Lys27 trimethylated H3 (H3K27me3; catalog no. 07-449) were obtained from Upstate Biotechnology. Immunoprecipitated samples with anti-histone H3 antibody and immunoglobulin G precipitation were used as positive and negative controls respectively for precipitations with specific antibodies in each experiment.

Chromatin of cultured cells was treated with formaldehyde to cross-link DNA to protein *in situ* and sonicated to an average size of 0.5 kb. Sonicated chromatin was then diluted 10-fold in ChIP dilution buffer (200 μl), precleared with 80 μl salmon sperm DNA/protein A agarose for 1 h at 4 °C, with rotation. A portion of the