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Immortalization and characterization of mouse temporomandibular joint disc cell clones with capacity for multi-lineage differentiation

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SUMMARY

Objective: Despite the importance of temporomandibular joint (TMJ) disc in normal function and disease, studying the responses of its cells has been complicated by the lack of adequate characterization of the cell subtypes. The purpose of our investigation was to immortalize, clone, characterize and determine the multi-lineage potential of mouse TMJ disc cells.
Design: Cells from 12-week-old female mice were cultured and immortalized by stable transfection with human telomerase reverse transcriptase (hTERT). The immortalized cell clones were phenotyped for fibroblast- or chondrocyte-like characteristics and ability to undergo adipocytic, osteoblastic and chondrocytic differentiation.
Results: Of 36 isolated clones, four demonstrated successful immortalization and maintenance of stable protein expression for up to 50 passages. Two clones each were initially characterized as fibroblast-like and chondrocyte-like on the basis of cell morphology and growth rate. Further the chondrocyte-like clones had higher mRNA expression levels of cartilage oligomeric matrix protein (COMP) (>3.5-fold), collagen X (>11-fold), collagen II expression (2-fold) and collagen II:I ratio than the fibroblast-like clones. In contrast, the fibroblast-like clones had higher mRNA expression level of vimentin (>1.5-fold), and fibroblastic specific protein 1 (>2.5-fold) than the chondrocyte-like clones. Both cell types retained multi-lineage potential as demonstrated by their capacity to undergo robust adipogenic, osteogenic and chondrogenic differentiation.
Conclusions: These studies are the first to immortalize TMJ disc cells and characterize chondrocyte-like and fibroblast-like clones with retained multi-differentiation potential that would be a valuable resource in studies to dissect the behavior of specific cell types in health and disease and for tissue engineering.

Introduction

Temporomandibular joint disorders (TMJDs) are a term encompassing a spectrum of clinical signs and symptoms, which involve the masticatory musculature, the temporomandibular joint (TMJ) and associated structures. These disorders often present with limitation or deviation in mandibular motion, open or closed locking of the jaw, TMJ sounds including clicking, popping and crepitus, and pain in masticatory muscles, TMJ and the face as well as headaches[1,2]. TMJD symptoms occur in approximately 6–12% of the adult population in the United States[3]. Of the patients with TMJDs, approximately 80% present with signs and symptoms of joint disease including disc displacement, arthralgia, osteoarthrosis and osteoarthritis[4,5], indicating that an understanding of the underlying pathobiology of diseases of the joint, and more specifically of the contribution of the TMJ disc, could be beneficial towards potential targeted therapies for a large proportion of patients with TMJDs.

The TMJ disc is a tissue of substantial importance because of its role in normal joint function in permitting mandibular movements and because its degeneration leads to compromised joint function[6–9]. In humans, the TMJ and disc start to form during the first trimester as a mesenchymal condensate in the TMJ region[10,11]. The disc undergoes progressive pre- and post-natal differentiation as it matures from a ligamentous/myotendinous structure to a specialized fibrocartilaginous tissue in response to function[12–16].

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The TMJ disc transmits and dissipates loads acting on the joint and adapts its shape to changing geometry of the articular surfaces, thus minimizing small contact areas and local peak forces[9,17]. Because the disc is subjected to tensile, compressive and shear forces, it is configured for anisotropic mechanical behavior and has a matrix composition and organization designed to withstand these complex mechanical challenges[18–22]. These complex functional demands result in a heterogenous tissue with differences in the regional distribution of extracellular matrices and cell phenotypes. Thus the mature disc has a fibrous tendinous structure with fibroblastic cells at sites of tension, a primarily cartilaginous matrix and chondrocyte-like cells at sites of compression and a mixed tissue composition and intermediate fibrochondrocytic cells where the tissue is subjected to complex tensile, compressive and shear forces[12,20,23–27]. Ultimately, this structural organization and composition of the disc is determined by the cells contained within the tissue.

Despite the importance of TMJ disc cells to the disc's integrity, contributions to normal joint function and role in disease progression, studying their responses to physiologic, pathologic biological and mechanical cues has been complicated by the lack of a detailed characterization of the cell subtypes comprising the disc. Furthermore, even though the disc is known to contain diverse cell phenotypes, the fundamental differences between fibroblast-like or chondrocyte-like cells or other putative cell types have not been elucidated. The longer-term aim of our studies is to elucidate the key characteristics of TMJ disc cell subtypes and their specific responses to exogenous and endogenous stimuli. The specific goals of the present study were to immortalize, clone and characterize mouse TMJ disc cells that primarily demonstrate either fibroblast-like or chondrocyte-like phenotypes and determine whether these clones retain the capacity for multi-lineage differentiation. We chose to establish immortalized cell clones from mice TMJ discs both to develop a needed resource to undertake *in vitro* mechanistic studies, and to establish protocols for subsequently immortalizing the less readily available human cells. The immortalization, cloning and characterization of these cells provide a valuable resource for future studies to determine cell type-specific responses to physiologic or pathologic cues that could offer critical insights on disease progression, prevention and treatments including tissue engineering of the TMJ disc.

Materials & methods

Reagents and animals

All cell culture reagents and media were purchased from Invitrogen Corp. (Carlsbad, CA) and chemicals were from Sigma–Aldrich Corp. (St. Louis, MO) unless otherwise mentioned. Total RNA extraction kit was from Qiagen Corp. (Valencia, CA) and quantitative real time reverse-transcriptional polymerase chain reaction (qRT-PCR) kits were obtained from Applied Biosystems (Carlsbad, CA). Bicinchroninic acid assay (BCA) protein assay kit was purchased from Thermo Scientific (Rockford, IL). Transfection agent Fugene HD, hygromycin B, α-MEM, 10% fetal bovine serum (FBS), fungizone and antibiotics were purchased from Invitrogen (Grand Island, NY). Primary antibodies to mouse vimentin, cartilage oligomeric matrix protein (COMP), and Collagen X were from Abcam Plc. (Cambridge, MA), to mouse fibroblast specific protein 1 (FSP1), aggrecan and β-actin were from Sigma–Aldrich Corp., and to mouse collagen I and collagen II were from EMD Chemicals Inc. (Gibstown, NJ). The pGRN145 plasmid containing a cDNA encoding human telomerase reverse transcriptase (hTERT) was obtained from ATCC (Manassas, VA). C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animal procedures were conducted in compliance with federal and institutional guidelines and approved by the Institutional Animal Care and Use Committee.

Determination of in vivo disc cell phenotypic proportions and distribution

For histological analyses, mice heads or knees were fixed in 4% paraformaldehyde, decalcified with 10% of ethylenediaminetetraacetic acid, embedded in paraffin, 5 μm thick sections cut and stained with hematoxylin and eosin. Cell numbers by phenotypes and distribution were quantified from tissue sections from three mice. Cells demonstrating elongated, narrow spindle shaped appearance were morphologically classified as fibroblast-like, while those displaying a rounded morphology with lacunae were classified as chondrocyte-like.

Isolation and immortalization mouse TMJ disc cells

TMJ discs from 12-week-old female mice were retrieved following euthanasia and cultured as described previously[28,29]. The discs were washed with phosphate buffered saline (PBS) containing antibiotics and fungizone, minced and incubated with α-MEM containing 10% FBS, and 100 units/ml of streptomycin and penicillin for 2–4 weeks. Passage two cells were immortalized by stable transfection with the vector pGNR145 expressing hTERT cDNA using Fugene HD. The transfected cells were selected in presence of hygromycin B (35 μg/ml) over 5 weeks and positive clones were subcultured in medium with hygromycin B (10 μg/ml).

Determination of cell immortalization

Of 36 isolated clones, four demonstrated successful immortalization as determined by telomerase assays and the ability to maintain expression of select markers for up to 50 passages. Telomerase activity was assessed using a telomere repeat amplification protocol (TRAP) kit (Roche, Mannheim, Germany). The TRAP assay involves a two-step process in which the telomerase-mediated elongation products are first amplified by PCR using GeneAmp PCR System 9600 (Applied Biosystems). Samples with enzyme-inactivation by heat treatment of the cell extract for 10 min at 85°C prior to the TRAP reaction served as negative controls. The amplified products were quantitated by readings at an absorbance wavelength of A_{450nm} against a blank with a reference wavelength of A_{690nm}. The amplified ladder was visualized following electrophoresis on a 12.5% polyacrylamide gel and staining with ethidium bromide. The stability of protein expression for versican and aggrecan previously shown to be expressed in the TMJ disc[15,30] was determined by Western blots.

Cell characterization by morphology, growth rate and specific markers

Morphological characteristics of the cells were assessed at days 1 and 8 of culture by phase contrast microscope (Nikon TS100). For cell growth assays, the clones were seeded at a density of 1.5×10^4 cells in 100 mm culture dish in α-MEM and cells counted at 2, 4, 6, 8 and 10 days. Passage-dependent expression of specific proteins was assayed for cells at passages 2–50, with about two population doublings corresponding to one passage.

To assay for fibroblast-like or chondrocyte-like markers, the clones were cultured in media supplemented with 10% FBS and hygromycin B (10 μg/ml) for 2 days followed by incubation without hygromycin until the cells were ~80% confluent. The cells were fixed for immunocytochemistry, and the expression of markers

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