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Transcriptional regulation of Proteoglycan 4 (PRG4) by 17 β -estradiol in immortalized baboon temporomandibular joint disc cells

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Abstract

Temporomandibular joint disorders (TMDs) affect a significant portion of the U.S. population with the majority seeking treatment being women of childbearing age. Due to this striking sexual dimorphism it has been postulated that there is a role for sex hormones in the maintenance of normal temporomandibular joint (TMJ) function. Proteoglycan 4 (PRG4) is a secreted lubricating molecule required for maintaining low frictional levels within articular joints, however its role in the TMJ is not well characterized. In this study we describe the development of immortalized baboon cells isolated from specific regions of the TMJ disc and their use in the investigation of PRG4 expression and localization patterns in the TMJ. We identified conserved estrogen response elements within the 5' flanking region of the PRG4 gene of several species, and found that treatment of baboon TMJ disc cells with estrogen led to reduced PRG4 promoter activity and mRNA expression *in vitro*. The observed negative regulation of PRG4 by estrogen could lead to increased friction and degradation of joint components over time. This study, for the first time, provides evidence of the regulatory potential of estrogen on PRG4 gene expression and suggests a novel etiology for the gender disparity observed among TMD patients.

Keywords

Temporomandibular joint; proteoglycan 4; extracellular matrix; estrogen; alternative splicing; cartilage

Unique to mammals, the temporomandibular joint (TMJ) is a synovial joint located at the site of articulation between the mandible and cranial base. An interarticular disc lies between the mandibular condyle and the glenoid fossa of the temporal bone, creating upper and lower compartments that allow for the joint's complex movement. The disc is comprised of a heterogeneous population of fibroblasts, fibrochondrocytes and chondrocytes that form a dense, fibrous surface for the condylar head to slide across, thereby decreasing pressure-loads and increasing the efficiency of the joint (1). Encapsulating the joint is a synovial membrane lined with cells that secrete synovial fluid, creating a liquid environment

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Conflicts of interest

The authors report no conflicts of interest.

responsible for providing nutrition and lubrication to the joint. A network of factors maintains homeostasis of the TMJ, and disruption at any level can lead to pathological conditions affecting the joint.

Temporomandibular joint disorders (TMDs) encompass a wide range of symptoms involving the TMJ and its associated structures. Symptoms include crepitation of the joint, limited range of joint movement and facial pain. An estimated 15% of the adult population in North America seeks treatment for TMJ disorders; however 80% of those are women predominately of reproductive age (2–4). This striking sexual dimorphism has prompted numerous studies geared at delineating the link of sex hormones to TMDs. Several key findings point to a role for estrogen in TMJ maintenance and metabolism, including early studies identifying functional estrogen receptors in the TMJs of female but not male baboons (5, 6). Animal studies have shown the relationship between estrogen and TMJ remodeling by treating rats with exogenous estrogen; as estrogen doses increased, so did abnormal changes in collagen and protein content within the TMJ (7, 8). Epidemiological studies showing that postmenopausal women utilizing exogenous hormone therapy had considerably higher rates of developing TMDs compared to those not exposed to exogenous hormones also indicate that the TMJ may serve as a target tissue for estrogen regulation (2). Histological analyses for the presence of estrogen receptors in human TMJ discs identified more receptors in females compared to males, suggesting the TMJ may be a sex hormone target tissue (9). These epidemiological reports documenting clinical observations of sexual disparity in TMD patients paired with studies identifying the presence of functional estrogen receptors in the TMJ complex of female baboons provide compelling evidence suggesting the involvement of estrogen, whether directly or indirectly, in the pathogenesis of TMDs. Finally, recent data suggests that a defect in the lubricating system of the joint may be responsible for the pathogenesis of TMDs leading to the development of degenerative TMJ disease (10, 11).

Proteoglycan 4 (PRG4), also known as lubricin, megakaryocyte stimulating factor and superficial zone protein, is a secreted extracellular matrix molecule produced by the synoviocytes and superficial zone chondrocytes of articular joints. The primary function of PRG4 is to provide boundary lubrication to articular surfaces of joints, thereby reducing frictional deterioration of joint tissues. Alternative splicing of the 12 exons comprising the PRG4 gene results in at least four known splice variants. Exons 2, 4 and 5 are alternatively spliced, while the central, mucin-like domain encoded by exon 6 provides the cues for extensive o-linked glycosylation necessary to generate the protein's hydrorepulsive properties that are responsible for boundary lubrication. *In vitro* frictional studies have demonstrated the ability of PRG4 to lubricate two shearing cartilage surfaces, cartilage and glass, and latex and glass as effectively as whole synovial fluid (SF), with proteolytic digestion of SF resulting in diminished lubricating ability (12).

The significance of PRG4 in joint biology is demonstrated by the discovery that any one of at least 13 different mutations in the 3' end of the gene leads to a shortened PRG4 protein causative in the autosomal recessive disorder camptodactyly-arthropathy-coxa vara-pericarditis syndrome (CACP; OMIM: 208250) (13, 14). Patients afflicted with CACP have normal appearing joints at birth but quickly develop precocious joint failure, the hallmark

feature of which are flexion contractures of the phalangeal joints of the fingers and toes and arthropathy, or restricted movement and swelling of the larger joints due to noninflammatory synovial hyperplasia (15, 16). Recently, a PRG4 knockout mouse has been generated and exhibits the same phenotype as that seen in patients with CACP, including reduced joint lubrication and synovial overgrowth when compared to wild-type mice (17, 18). While these studies clearly indicate that PRG4 is required for normal joint homeostasis, its role in TMJ physiology remains to be determined.

Due to the vast diversity of TMJ morphology and anatomy among species it has been difficult to perfectly mimic the anatomy of the human TMJ. Researchers have used a variety of animal models for TMJ research, including rabbits, dogs, sheep, pigs, cattle, goats, rodents, and non-human primates (19, 20). For our research we have chosen to use the olive baboon, *Papio hamadryas anubis*, due to its more similar TMJ anatomy to humans. In an effort to better understand the molecular mechanisms underlying normal TMJ homeostasis, we describe the development and characterization of immortalized, baboon TMJ cells derived from the lateral and central portions of the disc. The TMJ disc is oftentimes the origin of failure within the TMJ complex; therefore, developing disc cell lines is an important step in understanding the contribution of PRG4 in maintaining normal joint homeostasis. By characterizing the full complement of PRG4 variants expressed by TMJ disc cells we begin to provide a foundation to understand the role of PRG4 in the TMJ and its potential involvement in the pathogenesis of TMDs. Our identification of several estrogen response elements (EREs) within the promoter of the PRG4 gene suggests that PRG4 may serve as an estrogen target gene. To investigate the regulatory potential of estrogen on PRG4 gene transcription we utilized the immortalized baboon TMJ cells to perform *in vitro* promoter studies. Determining the ability of estrogen to influence PRG4 transcriptional events is critical for elucidating the etiologies of TMD. Our results demonstrate that estrogen negatively regulates PRG4 gene transcription in TMJ disc cells. This study provides, for the first time, evidence of the ability of estrogen to repress PRG4 gene expression, and suggests a novel mechanism that may explain, in part, the pronounced sexual disparity observed among TMD patients.

Material and Methods

Baboon TMJ discs

TMJ discs were dissected under sterile conditions from a five-year old, female baboon obtained from the Southwest National Primate Research Center (San Antonio, TX, USA). For immunochemistry, discs were snap frozen in liquid nitrogen and stored at -80°C prior to cryosectioning. To establish primary cultures, explants of the central and lateral portions of the disc were minced and placed in culture plates flooded with DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologics, Lawrenceville, GA, USA), 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen). After outgrowth was established, the cells were incubated until the monolayer achieved 50% confluency then detached with trypsin and subcultured.

Retroviral production and infection of TMJ disc cells

Production of the amphotropic hTERT retroviral vector containing the human telomerase gene and puromycin selection marker, pBABE-puro-hTERT (Addgene, Cambridge, MA, USA), was performed as described previously (21, 22). Briefly, the amphotropic packaging cell line, Phoenix-Ampho cells (Orbigen, San Diego, CA, USA), was grown in DMEM until semi-confluency. The cells were transiently transfected using Fugene 6 (Roche, Boulder, CO, USA) and the retroviral vector pBABE-puro-hTERT. The dishes were returned to the incubator and conditioned medium was harvested at 48 h. Primary disc cells were subcultured until 40% confluent. After aspirating the original medium, 5 ml of 0.45 μ m-filtered retroviral supernatant containing 4 mg/ml polybrene was added. After 3 h, 5 ml of DMEM was added and cells were incubated for 12 h followed by replacement of retroviral medium with fresh DMEM. The cells were allowed to recover for 24 h then infected cells selected using 3 μ g/ml puromycin (Sigma, St. Louis, MO, USA). Cells used in this study were limited to passage 3 or earlier to maintain any chondrocytic phenotypes.

Cell growth curve and telomerase expression

Population doubling times of immortalized TMJ cells (passage one) were determined by seeding 5×10^3 cells per well in 24-well tissue culture plates. Cells were allowed to attach overnight then cells in duplicate wells were counted daily for 2 wk. A cell proliferation curve determined doubling time. Telomerase expression by immortalized cells was monitored using the telomerase primers (sense, 5'-CTGCCTGAAGGAGCTGGT-3'; antisense, 5'-ACCGTGTGGGCAGGTAG-3') and the following PCR conditions: initial denaturation 5 min at 95°C, followed by 35 cycles of denaturation (30 s at 95°C), annealing (30 s at 58°C for PRG4 and 60°C for Cyclo A), and extension (1 min at 72°C) concluding with a final extension for 7 min at 72°C. PCR products were resolved on 1.5% agarose gels, stained with ethidium bromide and visualized under ultraviolet light.

Antibody generation

A region of the conserved PRG4 sequence corresponding to exons 7-12 was recombinantly expressed in *E. coli* as a six-histidine tagged fusion protein. The recombinant protein was used as an immunogen to produce rabbit polyclonal anti-PRG4 antibody (Bethyl Laboratories, Montgomery, TX, USA). The antibody was characterized using Western blot analyses and immunoprecipitation (23).

Immunocytochemistry

Disc cells were cultured on coverslips, fixed with ice-cold acetone and blocked with 1% normal goat serum and then incubated with either anti-PRG4 (1:500) or pre-immune serum (1:500). Primary antibody was detected by incubation with AlexaFluor 488, a FITC-conjugated, goat anti-rabbit secondary antibody (1:1000; Vector Laboratories, Burlingame, CA, USA). The cells were stained with DAPI, affixed to slides using Vectashield (Vector Laboratories) and images recorded using the Zeiss LSM 510 confocal microscope. Baboon TMJ discs were snap frozen, cryosectioned and 10- μ m thick sections fixed in 4% paraformaldehyde. The slides were then incubated with antibodies and confocal images recorded as described above.

RNA isolation and RT-PCR

Total RNA was isolated from disc cells (Qiagen, Valencia, CA, USA) and reverse transcribed (Applied Biosystems, Carlsbad, CA, USA). For analysis of PRG4 splice variants, templates were amplified using PRG4 primers spanning the splice region comprising exons 1-6 (sense, 5' CTGAAAACAACGATGGCATG-3'; antisense, 5' TCATCTACAACCTGGTGGTTTGG-3'). Cyclophilin A primers (sense, 5'-GGTGACTTCACACGCCATAA-3'; antisense, 5'-CATGGCCTCCACAATATTCA-3') served as the control. PCR conditions were as follows: initial denaturation at 95°C for 4 min; 35 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min; 72°C for 7 min. PCR products were resolved on 1.5% agarose gels, purified and identified by automated DNA sequencing.

Quantitative polymerase chain reaction (qPCR) analysis

To determine relative expression of the PRG4 splice variants, primers were designed to span splice junctions unique to each variant allowing for amplification of only the specific variant of interest (Table 1). In addition, a primer set was included to quantify total PRG4 expression by amplification of the highly conserved region of the gene, exons 10 and 11, which are included in all known splice variants. qPCR amplification of specific PRG4 splice transcripts, total PRG4 and the endogenous control, 18s rRNA, (sense, 5'-AGACCTGGAGCGACTGAAGA-3'; antisense, 5'-AGAAGTGACGCAGCCCTCTA-3') was performed for each template using Power SYBR® Green PCR Master Mix (Applied Biosystems) and the ABI 7300 Real-Time PCR System (Applied Biosystems) as follows: an initial 50°C for 2 min. followed by 95°C for 10 min. then 40 cycles of 95°C for 15 s, and 60°C for 1 min. All experiments were conducted independently at least three times with triplicate reactions for each cDNA tested and results were normalized to 18s rRNA using the C_t method. Statistical significance was determined by student's T test or two-way analyses of variance ($p < 0.05$).

PRG4 promoter analyses

An approximate 3 kb stretch of human nucleotide sequence upstream of the PRG4 transcriptional start site was entered into the algorithm AliBaba 2.1 (24) which localizes putative estrogen response elements (ERE) using the TRANSFAC (25) database of transcription factors. DNA sequence alignments between human, chimpanzee, orangutan, marmoset and gibbon promoter sequences were performed using ClustalW2 (26).

17 β -estradiol treatment

Immortalized baboon TMJ disc cells were seeded at 5×10^5 cells per well in six-well tissue culture plates in phenol red-free DMEM supplemented with 10% charcoal-stripped FBS. After cells reached 30% confluency, the medium was replaced with fresh phenol red-free DMEM plus 10% charcoal-tripped FBS either with or without 10 nM 17 β -estradiol (E2). Medium and E2 was replaced every 24 h and after 72 h; cells were harvested for RNA isolation, cDNA synthesis and qPCR.

PRG4 promoter-luciferase reporter gene constructs

PCR-generated PRG4 putative promoter fragments were digested with BglII and MluI to create compatible ends for insertion into the promoter-less reporter vector, pGL3-Basic (Promega, Madison, WI, USA). The pGL3-basic vector was also digested with the same restriction enzymes and the PRG4 fragments ligated. Competent *E. coli* JM109 bacterial cells (Promega) were transformed with ligation reactions, expanded and DNA collected via maxipreps. All clones were verified for promoter insertion and sequence.

Transient transfection and reporter gene assay

Twenty-four hours prior to transfection immortalized baboon central disc TMJ cells were seeded into 24-well plates at 5×10^5 cells per well. The cells were co-transfected at 50% confluency in triplicate with the pGL3+promoter construct, empty pGL3 plasmid, or a positive pGL3 control along with the pRL-TK plasmid, a renilla luciferase reporter vector to serve as a control for transfection efficiency. FuGene 6 (Roche) was used to transfect the cells according to the manufacturer's protocol. Post transfection, wells were treated with or without 10 nM E2 in phenol red-free DMEM plus 10% charcoal-tripped FBS with media and E2 replaced every 24 h. Cells were lysed at 48 h post-transfection and both the firefly and renilla luciferase activity monitored using the Dual Luciferase Assay kit (DLR; Promega) and GloMax-20/20 Single Tube Luminometer (Promega) per manufacturer's suggested protocol. Luciferase activity was normalized to renilla luciferase activity and all experiments were performed at least three times independently with triplicate samples for each. Statistical significance was determined using the student's T test.

Results

Generation of immortalized baboon TMJ disc cells

The hTERT immortalized lateral (LD) and central disc (CD) cells appear morphologically unchanged from their pre-immortalized state (Figs. 1A,B). Although we cannot conclude that there are morphological differences in cells isolated from different parts of the disc, overall the cells from each region exhibited an outward appearance of elongated, fibroblastic cells and rounder, chondrocytic-like cell shapes indicative of their fibrocartilage lineage (Figs. 1A,B). Cell proliferation assays indicated that CD cells proliferated at a slightly higher rate than LD cells (Fig. 1C). However at approximately day three in culture both cell types appeared to proliferate at a consistent rate with the population doubling approximately every 48 h. Telomerase expression by immortalized cells was monitored by amplification of telomerase transcripts by PCR with expression observed in immortalized cells but not primary disc cells (Fig. 1D). Infected cells continued to divide and maintain hTERT expression past 30 population doublings (data not shown).

Localization of PRG4 protein in immortalized baboon TMJ disc cells and whole discs

PRG4 protein expression by disc cells was evaluated using immunofluorescent staining and visualized with confocal microscopy. Confocal images of lateral and central disc cells documented diffuse cytoplasmic PRG4 protein expression (Figs. 2A,B). No staining was observed in cells incubated with pre-immune rabbit serum (Figs. 2C,D). PRG4 staining in

the lateral region of the disc displayed an amorphous, less dense staining (Fig. 2E) as compared to the more central portion of the disc (Fig. 2F). Intense staining was observed near the most superficial edges of the central region of the disc (Fig. 2F). No staining was observed in sections incubated with pre-immune serum (Figs. 2G,H).

PRG4 transcript expression in baboon TMJ disc cells

Amplification of PRG4 splice variants detected all four known isoforms (V_0 , V_1 , V_2 and V_3) in both cell types (Fig. 3A). To determine overall PRG4 expression levels in disc cells, qPCR analysis was performed using primers designed to amplify the conserved regions of the gene encoded by exons 10 and 11. The data revealed the overall expression of PRG4 in CD cells is two-fold higher than that expressed by LD cells ($p=0.0009$; Fig. 3B).

Estrogen response elements in the 5'-flanking region of the PRG4 gene

A putative estrogen response element (ERE) consensus sequence (5'-GGTCACaaCTACC-3') as well as three half-EREs (half of palindromic sequence) in the 3 kb sequence upstream of the PRG4 transcriptional start site were identified. The full ERE is located at position -1074 to -1087 relative to the transcriptional start site in the 5'-flanking region of the PRG4 gene. The three half EREs are at positions -1462 to 1467, -1218 to -1223 and -689 to -694 bp (Fig. 4A).

Conservation of ERE sequence in PRG4 promoter of multiple species

To determine if ERE consensus sequences were present in species other than human, the promoters from human, chimpanzee, orangutan, marmoset and gibbon were aligned using the transcription factor binding site prediction algorithm, ClustalW2. We found that the identical, imperfect ERE consensus sequence was present in the PRG4 gene promoters of human and non-human primates at the same approximate location (Fig. 4B).

Estradiol suppresses expression of the PRG4 gene

To determine if estradiol (E2) regulates overall expression of PRG4 transcription, cells were treated with 10 nM E2. This concentration was chosen based on reports that 10 nM E2 is sufficient to stimulate estrogen responsive genes in cultured cells (27-30). The qPCR analysis of total PRG4 expression showed that E2 significantly down-regulated transcription of the PRG4 gene in lateral and to a greater degree in central disc cells when compared to non-treated cells (LD $p=0.004$, CD $p=0.001$; Fig. 5A). Examination of individual splice variants in CD and LD cells for changes in expression levels post-E2 exposure found that full the expression of length transcripts of PRG4, V_0 ($p<0.01$) as well as V_1 ($p<0.001$) was attenuated in response to E2 exposure in both cell types relative to non-treated cells (Figs. 5A,B). Transcription of the variant V_2 ($p<0.05$) was reduced in only the central disc cells while V_3 transcripts were not significantly reduced in either cell type (Figs. 5A,B).

In Vitro promoter studies of E2 regulation of PRG4 in TMJ cells

Immortalized TMJ central disc cells transiently co-transfected with either the -2.3 kb prom +luc construct containing the full ERE and three half-EREs sites and the control construct pRL-TK or the -92 prom+luc construct with all ERE sites deleted demonstrated negative

regulation of the PRG4 promoter when exposed to estrogen. E2 negatively regulated the PRG4 promoter activity of the -2.3 kb promoter fragment in a statistically significant manner while the -92 promoter construct showed some down-regulation however it was not statistically significant (Fig. 6).

Discussion

Temporomandibular joint disorders affect a considerable portion of the American population with up to 35 million people enduring persistent, mild to debilitating facial pain. The articular disc is often the primary source for many of the problems associated with the joint, with approximately 70% of TMDs attributed to disc displacement (31). Failure of the lubricating system of the TMJ has been proposed as being the precipitating event leading to deterioration of the joint (10, 11). There is accumulating and compelling evidence that PRG4 is the primary lubricating molecule of articular joints and that it plays a critical role in joint homeostasis (32). PRG4 has been shown to be the major lubricating component of synovial joints by providing anti-adhesive properties to the surfaces it is in contact with and inhibiting synovial overgrowth that can impede joint mobility (18, 33–35). Moreover, recent studies indicate intra-articular expression of PRG4 protects against age-related osteoarthritis in synovial joints and acts as a friction-lowering lubricant at the cornea-eyelid interface (36, 37). To better understand the role of PRG4 in the lubrication system of the TMJ, we have established immortalized baboon TMJ disc cell lines derived from two regions of the articular disc, the most interior region and the outer, lateral edge of the disc (Fig. 1). These cells are expected to represent a heterogeneous population of cells in the TMJ and that comprise TMJ disc, which are principally fibroblasts and fibrochondrocytes (38). We did not make a distinction of phenotype loss, or whether both fibroblasts and fibrochondrocytes respond to E2 attenuation of PRG4 gene transcription. However, this study shows that at least one of the disc cell types is responsive to E2-mediated inhibition of PRG4 expression, offering a framework for additional investigations to characterize E2-responsive cells that result in a decrease in PRG4 expression. The immortalized cells were used here to provide a unique, renewable source of material with which to perform *in vitro* experiments and characterize the baseline expression profile of PRG4 splice variants in the articular TMJ disc.

The results of the first part of our study revealed regional differences in PRG4 expression in both CD and LD cells derived from the disc as well as in whole baboon TMJ disc tissue. Immortalized baboon TMJ cells in monolayer demonstrated diffuse, cytoplasmic localization of PRG4 (Fig. 2A,B). Interestingly, immunofluorescent staining of baboon TMJ whole disc sections indicated spatial differences in PRG4 expression with less staining observed in the more lateral areas and more intense staining in the central portion in particular, the most superficial edges of the central region (Fig. 2E,F). As the central region of the disc bears the majority of frictional forces during normal mandibular movement, these results appear to correspond with what others have seen in 3D chondrocyte scaffolds with increasing PRG4 expression in response to mechanical stimuli (39–41). Our observation of intense PRG4 staining in disc cells agrees with PRG4 localization examined in human TMJ discs with (18 discs) and without (34 discs) internal derangement; PRG4 was evident in the surface region of discs when compared to deeper, central regions (42, 43). Disc surface

enrichment for PRG4 is consistent with the observations that mechanical stimuli promote PRG4 expression, indicating that PRG4 plays a lubricating role at the site of articulation between surfaces of the disc and the cranial base, and the disc and mandible. The expression of PRG4 in the disc appears to be essential for optimal lubrication of the joint; however, further studies are needed to establish the exact nature of PRG4 in the TMJ and what interactions it may have with the surrounding extracellular matrix.

qPCR analysis of immortalized disc cells provided additional support of differential spatial PRG4 expression in the TMJ disc. We were able to isolate and identify all four known PRG4 splice variants (V_0 , V_1 , V_2 and V_3) in both cell types (Fig. 3A). Moreover, we found that CD cells express two-fold more PRG4 transcripts than LD cells (Fig. 3B). Several studies have demonstrated that mechanical loading regulates PRG4 metabolism in both cartilage explant cultures and chondrocyte-seeded scaffolds (39–41). In all instances, PRG4 expression was upregulated in response to mechanical stimuli. While we cannot assume that the baboon TMJ disc cells used in this study or the TMJ disc itself will respond in a similar manner to mechanical stimuli, our results appear to be in line with previous studies demonstrating increased PRG4 expression in cartilage and chondrocytes subjected to loading forces (39–41). As a result of these studies we have shown that PRG4, known to be the primary lubricant of synovial joints, is expressed in the TMJ and it is therefore reasonable to presume that it provides a similar function to the TMJ.

While it is widely believed that sex hormones predispose women to developing TMDs, the mechanism by which this occurs has yet to be determined. The identification of putative ERE binding sites within the PRG4 gene promoter supports our hypothesis that estrogen regulates expression of PRG4 (Fig. 4A). Furthermore, we have identified a conserved putative ERE sequence in the PRG4 gene 5'-flanking regions of multiple species including human and non-human primates lending credence to the regulatory potential of estrogen in PRG4 gene transcription (Fig. 4B). Taken together, these findings provide clues to suggest a hormone-related mechanism underlying the gender disparities observed in TMDs.

In the present study we submit preliminary data that 17 β -estradiol (E2), the most abundant form of estrogen in females, differentially regulates the activity of the PRG4 gene in TMJ cells at the transcriptional level. Immortal baboon TMJ cells prove to be a suitable model with which to conduct our *in vitro* studies due to their endogenous expression of both PRG4 and estrogen receptors α and β (data not shown). Parallel studies utilized quantitative PCR to monitor overall PRG4 mRNA expression in TMJ cells after E2 exposure compared to non-treated cells. TMJ cells exhibited negative regulation of PRG4 transcription in response to E2 exposure. Both lateral and central disc cells demonstrated a decrease in overall PRG4 transcript production (Fig. 5A). In order to characterize changes in the relative levels of alternatively spliced PRG4 transcripts expressed by cells stimulated with E2, we utilized qPCR and primers designed to specifically amplify each individual variant in CD and LD cells. Overall transcript abundance was reduced for all variants after E2 exposure in both cell types (Fig. 5A,B). Each of the variants has the central glycosylation domain encoded by exon 6. Therefore, the implication is that there is a collective reduction in the overall quantity of PRG4 available to provide lubrication to the TMJ. Utilizing conventional *in vitro* reporter assays, we further demonstrated E2-mediated changes in PRG4 transcription post-

estradiol exposure. The PRG4 promoter construct containing ERE sites showed reduced luciferase activity in E2-treated cells compared to non-treated cells (Fig. 6). Cells transfected with the minimum promoter-luciferase construct also demonstrated some promoter repression in response to E2 stimulation; however these results were not statistically significant. These data support our hypothesis that the PRG4 gene serves as a target for estrogen-mediated transcriptional regulation.

In summary, we have provided the first evidence that estrogen represses transcriptional activity of the PRG4 gene in TMJ disc cells *in vitro*. Though this is by no means a comprehensive study of the regulatory effects of estrogen on PRG4 gene transcription, it does suggest that the PRG4 gene is susceptible to regulation by estrogen. Future studies are aimed at determining if estrogen's ability to suppress transcription in TMJ disc cells translates into reduced synthesis of PRG4 thereby comprising the lubrication system of the joint. Over time, a reduction in PRG4 protein synthesis would create an environment of increased friction and ultimately lead to degradation of joint components. Additionally, the knowledge that PRG4 is expressed in the TMJ and provides boundary lubrication to articular joints paired with the sexual dimorphism observed clinically in TMD patients provides compelling evidence that PRG4 may be involved in the pathogenesis of TMDs.

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Figure 1.

Characterization of immortalized baboon lateral and central TMJ disc cells. Phase-contrast images showing morphology of cultured immortalized TMJ lateral disc (A) cells and central disc (B) cells. Cultures are comprised of a heterogeneous mixture of fibroblasts and fibrochondrocytes. Growth curve shows cells double in population approximately every 48 hours with LD cells growing at a slightly slower rate than CD cells (C). PCR analysis of telomerase expression (D) in primary and immortalized baboon TMJ disc cells indicates immortalized cells maintain expression of the hTERT gene past 30 passages. (Lane 1 = primary CD cells, 2 = primary LD cells, 3 = immortalized CD cells, 4 = immortalized LD cells)

Figure 2.

Localization of PRG4 protein in immortalized TMJ cells and whole baboon discs. PRG4 expression by immortalized lateral (A) and central disc (B) cells was observed throughout the cytoplasm of both cell types (green) while lateral (C) and central disc (D) cells did not show PRG4 staining in negative controls. Immunohistochemical detection of PRG4 in whole baboon TMJ disc sections showed the most lateral region of the disc (E) exhibited less intense PRG4 (E) staining while the central portion of the disc (F) demonstrated PRG4 staining with intense staining at the most superficial edges. Sequential sections incubated with pre-immune serum did not indicate any PRG4 staining (G and H). Bar = 20 µm

Figure 3.

PRG4 transcript expression by immortalized baboon TMJ cells. Standard PCR (A) detected the presence of all four known PRG4 splice variants (V₀, V₁, V₂ and V₃). Variants were confirmed via DNA sequence analyses. The qPCR analysis (B) demonstrated overall PRG4 expression is two-fold higher in the cells isolated from the central portion of the disc as compared to the more lateral region of the disc (***p*=0.0009).

Figure 4.

Nucleotide sequence of PRG4 5' flanking region and ortholog alignment of putative ERE site. The full-length ERE sequence is indicated by a box at position –1074 to –1087, relative to transcriptional start site. Three half EREs at positions –1462 to 1467, –1218 to –1223 and –689 to –694 are underlined (A). Alignment of human and non-human primate putative full-length ERE sites contain similar, and in some cases identical, sequences within their respective PRG4 promoters. Red nucleotides represent conserved ERE sequence among species (B).

Figure 5.

qPCR analyses of PRG4 expression in estrogen treated disc cells. PRG4 expression was significantly reduced by both LD and CD cells exposed to E2 (A; LD $p=0.004$, CD $p=0.001$). PRG4 expression analyses of individual variants demonstrated a reduction in V₀ ($p<0.01$) and V₁ ($p<0.001$) in both cell types but V₂ ($p<0.05$) was reduced only in LD cells (B and C). V₃ was not significantly reduced in either cell type.

Figure 6.

Luciferase activity in estrogen-treated disc cells transfected with PRG4 promoter fragments containing ERE sites. -2.3 kb prom+luc construct contains the full-ERE (oval) and three half-ERE sites (triangles) while the -92 bp prom+luc does not contain any ERE sites. Estrogen treatment negatively regulates PRG4 -2.3kb promoter activity in transfected cells treated with E2 (black bars) compared to non-treated (grey bars) cells. The luciferase values are relative luciferase units (RLU) and represent the mean values for three independent experiments performed in triplicate (** $p < 0.005$).

Table 1

Primer sequences for quantification of splice variants. Primers were designed to amplify individual PRG4 variants, V₀, V₁, V₂ and V₃ by spanning unique splice junctions. Total PRG4 primers were designed to amplify all forms of PRG4 by utilizing the conserved nucleotide sequence from exons 10 and 11.

Primer sequences for quantification of splice variants		
Splice Variant	Sense primer (5'→3')	Antisense primer (5'→3')
V ₀	GGAATAACAGAAGAACATTCTG	TCATCTACAAC TGGTGGTTTGG
V ₁	TCGACTGTGGAGCTTTCCTG	GTTATCTTTTCCTTCTGTTA
V ₂	GCCACCTGCAACTGTGATTA	GTTATCTTTTCCTTCTGCAC
V ₃	TCATCTCAAGAGCTTTCCTG	GTTATCTTTTCCTTCTGCAC
Total PRG4	GGACCTTCTCAAACACACACC	TTCTGATGTTGGGCAGTGAT