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Continuous Passive Motion Applied to Whole Joints Stimulates Chondrocyte Biosynthesis of PRG4

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Abstract

Continuous passive motion (CPM) is currently a part of patient rehabilitation regimens after a variety of orthopedic surgical procedures. While CPM can enhance the joint healing process, the direct effects of CPM on cartilage metabolism remain unknown. Recent *in vivo* and *in vitro* observations suggest that mechanical stimuli can regulate articular cartilage metabolism of proteoglycan 4 (PRG4), a putative lubricating and chondroprotective molecule found in synovial fluid and at the articular cartilage surface.

Objectives—1) Determine the topographical variation in intrinsic cartilage PRG4 secretion. 2) Apply a CPM device to whole joints in bioreactors and assess effects of CPM on PRG4 biosynthesis.

Methods—A bioreactor was developed to apply CPM to bovine stifle joints *in vitro*. Effects of 24 hours of CPM on PRG4 biosynthesis were determined.

Results—PRG4 secretion rate varied markedly over the joint surface. Rehabilitative joint motion applied in the form of CPM regulated PRG4 biosynthesis, in a manner dependent on the duty cycle of cartilage sliding against opposing tissues. Specifically, in certain regions of the femoral condyle that were continuously or intermittently sliding against meniscus and tibial cartilage during CPM, chondrocyte PRG4 synthesis was higher with CPM than without.

Conclusions—Rehabilitative joint motion, applied in the form of CPM, stimulates chondrocyte PRG4 metabolism. The stimulation of PRG4 synthesis is one mechanism by which CPM may benefit cartilage and joint health in post-operative rehabilitation.

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INTRODUCTION

Articular cartilage functions to provide a low-friction, load-bearing surface which allows the bones of diarthrodial joints to slide smoothly against each other while transmitting load. Cartilage tissue has classically been divided into three zones: superficial, middle, and deep, with distinct biochemical content and organization that impart specific functions to each zone. For example, chondrocytes of the superficial zone of cartilage secrete specialized molecules, encoded by the proteoglycan 4 gene (PRG4, GenBank Accession Numbers: AF056218 for bovine partial sequence, U70136 for complete human sequence, also termed lubricin, SZP, CACP) [1–3], that are not expressed by chondrocytes in the deeper zone[1]. These PRG4 molecules mediate, at least in part, the boundary lubrication function of the articular cartilage superficial zone[4–7], and have been suggested to protect the cartilage surface by preventing cellular adhesion to the surface[2]. Mutations in the PRG4 gene cause camptodactyly-arthropathy-coxa vara-pericarditis (CACP) disease, which results in early onset non-inflammatory joint failure[8–10], demonstrating the functional importance of the gene *in vivo*.

Recent experimental observations support the hypothesis that mechanical stimuli play a role in regulating PRG4 expression in articular cartilage *in vivo*. During embryonic development of the mouse elbow joint, PRG4 mRNA expression begins at the onset of joint cavitation[10], suggesting that PRG4 expression might be induced by the initiation of relative motion between the articular surfaces. A similar pattern is seen during post-natal growth, where fetal bovine cartilage exhibits inconsistent PRG4 expression by chondrocytes near the articular surface, in contrast with adult tissue, which has abundant PRG4-expressing cells near the surface[11]. In both cases (*in utero* and *in vivo*), increased chondrocyte expression of PRG4 coincides with increased joint motion. Furthermore, abnormal mechanical stimuli may result in decreased PRG4 expression. In a meniscectomy-induced (i.e., mechanically induced) osteoarthritis model in sheep, abnormal joint motion resulted in degeneration of articular cartilage in certain regions of the tibial plateau, with decreased PRG4 expression in these regions[12]. PRG4 expression was also higher in covered (by meniscus) than uncovered regions for tibial plateaus of normal ovine joints, suggesting that site-associated variations in intrinsic PRG4 expression could be due to mechanical factors as well[12].

Other studies have demonstrated that certain mechanical stimuli can regulate PRG4 metabolism by chondrocytes cultured in various configurations *in vitro*. Dynamic surface motion applied to chondrocytes embedded in polyurethane constructs[13], and cyclic tensile strain applied to chondrocytes in alginate constructs[14] resulted in increased PRG4 mRNA expression. Similarly, certain magnitudes of static and dynamic compression[15] and dynamic tissue shear[16] applied to cartilage explants altered PRG4 proteoglycan secretion relative to that of unloaded controls, both during loading and following unloading. Such *in vitro* studies allow the application of well defined mechanical stimuli under controlled biochemical environments by eliminating a variety of systemic factors present *in vivo* that affect chondrocyte metabolism. However, even in cylindrical cartilage explants, where chondrocytes are still embedded in their native matrix, the micro-environmental phenomena (fluid flow, cell and matrix deformation) resulting from *in vitro* mechanical loading may be quite different from those experienced by intact articular cartilage in its native configuration covering bones, under physiological joint motion.

Continuous passive motion (CPM) was originally proposed as a therapeutic alternative to the traditional rehabilitative practice of joint immobilization for many orthopedic disorders and injuries[17]. CPM stimulation utilizes an external motorized device to move joints passively through a specified range of motion[18]. CPM is currently applied post-operatively to enhance patient recovery after anterior cruciate ligament reconstruction[19, 20]. CPM was also found in experimental studies to promote healing of articular cartilage defects[17, 21], and a variety of

other joint afflictions[22], supporting its use in post-operative rehabilitation of cartilage defect repair by autologous chondrocyte implantation[23, 24] and microfracture[25]. However, the direct effects of CPM on chondrocyte metabolism remain unknown.

The hypothesis tested here was that a CPM device could be used to stimulate a whole joint during culture, and that this rehabilitative motion, applied to cartilage in its native configuration, regulates chondrocyte metabolism. The objectives of this study were to a) determine the spatial variation in intrinsic cartilage PRG4 secretion before bioreactor joint culture, and b) determine effects of CPM on PRG4 biosynthesis using a bioreactor for culture of whole joints.

METHODS

Joint Isolation

Immature (1–3 wk) bovine stifle joints were obtained from an abattoir. Soft tissues were cleared away with a scalpel, and the femur and tibia bones were cut with a bone saw such that the total length of the extended joint was ~25 cm. The contents of the bone marrow cavity were removed with a curette and the cavity was washed 3 times with 3% hydrogen peroxide. The outside of the joint was then scrubbed with 3% hydrogen peroxide and patted dry with gauze pads. The following steps were performed using aseptic technique, and all materials used, including dissection surface, were initially sterile. The joint was scrubbed with sponges soaked with 7.5% povidone-iodine solution, and patted dry again. Under continuous irrigation with phosphate buffered saline with penicillin-streptomycin-fungizone, some joint tissues (muscle, periosteum, patella, patellar tendon, synovium, and fat) were cleared away using surgical scissors, a scalpel, and a periosteal elevator, taking care to leave the joint capsule intact until the end of this procedure, at which point it was opened and removed. The joint tissues remaining for bioreactor culture were the femoral and tibial bones and associated articular cartilage, the ligaments necessary to provide joint stability (anterior and posterior cruciate, lateral and medial collateral), and lateral and medial menisci.

Bioreactor Culture of Whole Joints & CPM Stimulation (Fig. 1)

The cut ends of the bones were secured into custom designed polysulphone bone clamps, and a flexible, autoclavable polypropylene enclosure was placed around the joint and sealed tightly around the bone clamps to maintain a closed, sterile environment for the joint. Tygon tubing (Cole Parmer®) connected the bioreactor environment to a reservoir containing 2 L of culture medium (low-glucose Dulbecco's modified Eagle's medium (DMEM), 10 mM HEPES buffer, 0.1 mM non-essential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) supplemented with 5% fetal bovine serum (FBS) and 25 μg/ml ascorbate. The bioreactor was filled with medium from the reservoir using a 3 channel high-flow rate peristaltic pump (Wiz Model HF, Teledyne Isco Inc.), operating at 83 ml/min, which also maintained medium circulation between the reservoir and the bioreactor throughout the duration of joint culture. The medium reservoir was submerged in a water bath to maintain the bioreactor temperature at 37°C and humidified 95% air/5% CO_2 was pumped through a 0.22 μm sterile filter into the reservoir to maintain the medium at pH 7.4. The bioreactor was adapted for attachment to a CPM device (FlexMate K500, BREG, Inc.), which can apply joint motion ranging between −10° and 120° of flexion, at rates between 30°/min and 150°/min.

For the experiments described here, CPM stimulation consisted of 24 h of continuous motion, with the joint oscillating between 10° and 46° of flexion (Fig. 1C) at 110°/min (43 s per cycle, or ~0.025 Hz). Intrinsic tension in the 4 intact ligaments maintained the articulating surfaces of the joint in contact, and no additional axial load was applied. Since the patella was discarded

during dissection, cartilage from the patellofemoral groove (PFG) was not in direct contact with other tissues during bioreactor culture with or without continuous passive motion stimulation. In contrast, certain regions of the lateral and medial femoral condyles (LFC and MFC) slide against the adjacent menisci and tibial plateau cartilage during CPM stimulation. Thus cartilage from the PFG was in a similar mechanical environment for joints in both bioreactor cultures, with or without CPM, and metabolism of chondrocytes in this region was expected to be independent of CPM stimulation. Cartilage from the LFC and MFC of the joints cultured with CPM was compared to cartilage from those regions of joints cultured without CPM to determine direct effects of rehabilitative motion on chondrocyte health. The LFC and MFC were further divided into three sub-regions based on the type of contact the sub-region had with adjacent tibial cartilage and meniscus during the motion protocol. These sub-regions were designated as continuously, intermittently, or never sliding against the adjacent tissues, determined for each joint by visually determining contact areas at maximum and minimum applied flexion angles, and this effect is referred to herein as sliding duty cycle (Fig. 1D).

Experimental Design (Fig. 2)

Experiment I: Fresh Joints—Some joints were used to determine the intrinsic level of PRG4 secretion without bioreactor culture. Following isolation of these joints (n=4, from different animals), cylindrical cartilage disks (3 mm diameter, ~0.5 mm thick) were explanted from 12, 29, and 36 sites on the PFG, LFC, and MFC, respectively (sites shown in Fig. 3B), and cultured for 2 days in 48-well tissue culture plates and incubators. Medium (as described above, but with 10% FBS, 0.5 ml per disk) was collected and replaced daily for analysis of PRG4 secretion. Control studies in which cartilage disks from these locations were cultured for 4 days showed that the site-associated pattern in PRG4 secretion (relative secretion among sites) was somewhat variable during day 1, but similar during each of the subsequent 3 days in culture; thus disks were cultured for 2 days to determine differences in secretion values among explant sites. For this reason PRG4 secretion over the first 2 days of culture is presented here for comparison of results of experiment I with those of experiment II.

Experiment II: Bioreactor-Cultured Joints—Paired stifle joints from each of 4 animals were isolated as described above, and transferred to bioreactors for whole joints. One bioreactor was attached to the CPM device, while the other was placed on a bench-top for culture without mechanical stimulation. Following 24 h of joint culture in bioreactors, cartilage disks were explanted using aseptic technique from 24 sites on the PFG, and 30 sites each on the LFC and MFC (sites shown in Fig. 3C). Sites on the femoral condyles were chosen to represent the 3 sliding duty cycle conditions (continuously, intermittently, and never sliding against another tissue, Fig. 3A). Each cartilage disk was analyzed for PRG4 immunolocalization within chondrocytes (4 disks per region) or PRG4 secretion during 2 days of culture in medium with 10% FBS, as in experiment I (12–18 per region).

Cartilage Analysis

PRG4 Immunolocalization—The presence of PRG4 within chondrocytes was determined qualitatively from cartilage disks that were incubated for 4 h following bioreactor culture in medium supplemented with 1 μM monensin, essentially as described previously; such treatment with monensin blocks PRG4 secretion and thereby enhances the detection of cells expressing PRG4 at high levels during the 4 h time period[26]. Upon termination of culture, the disks were frozen in Tissue Tek OCT (Sakura USA) and sectioned (5 μm slices) perpendicular to the articular surface. The sections were reacted with mAb 3-A-4 (a generous gift from Dr. Bruce Caterson, University of Wales, Cardiff, UK[11]), and detected with a peroxidase-based system (Vector Labs). Stained samples were viewed to identify immunoreactive cells, indicating synthesis of PRG4. Adjacent sections probed with a non-specific mouse IgG antibody served as negative controls. Qualitative results were documented by photomicroscopy

using brightfield illumination. *PRG4 Secretion.* Culture medium was collected and replaced every 24 h for cartilage disks incubated for 2 days. As described previously[26], conditioned medium samples were quantitatively analyzed for PRG4 content by indirect ELISA using mAb 3-A-4. Briefly, samples were diluted serially, adsorbed, and then reacted with mAb 3-A-4, horseradish peroxidase-conjugated secondary antibody, and ABTS substrate, with 3 washes with PBS-0.1% Tween (Bio-Rad) between each step. A standard curve was generated from samples containing known amounts of PRG4, obtained from conditioned medium from explants from the superficial zone of bovine calf cartilage as previously described[1]. The protein-equivalent amount of PRG4 in each sample was calculated from the linear region of the standard curve (between 0.078 and 5 μg/ml of PRG4), as described previously[26, 27]. Control studies indicated that cartilage disks contained PRG4 in amounts, ~1 $\mu g/cm^2$, that were small relative to that secreted into the medium, so that the secreted quantities were representative of biosynthesis levels.

Control studies showed that cartilage viability, assessed by staining cartilage disks with Calcein-AM and ethidium homodimer-1 to localize live and dead cells, respectively, and quantified by image analysis with Matlab, was maintained during bioreactor culture, with or without CPM stimulation. Chondrocyte viability was high (80–93%) for all regions and loading conditions, and varied somewhat with joint region ($p=0.07$), but not in a CPM-dependent manner ($p=0.42$ for interaction between CPM stimulation and joint region).

Data Reduction

PRG4 Immunolocalization—Chondrocytes staining for PRG4 (PRG4+) as well as those not expressing PRG4 (PRG4-) were identified manually in a 300μm × 400μm area of each vertical section (one randomly chosen section from central region of each cartilage disk), and counted by image analysis with Matlab 7.0. Since the total number of cells varied with joint region, PRG4 expression was reported as a percentage of the total cell number in the area counted: % PRG4+ = (100% * # PRG4+ chondrocytes) / (#PRG4+ chondrocytes + #PRG4 −chondrocytes). *PRG4 Secretion.* PRG4 protein secretion was reported normalized to cartilage disk surface area, and was also represented as a contour plot. The contour plot was created using Matlab 7.0 to interpolate between input mean secretion values at the locations of each cartilage disk, and mapped onto the joint surface, with color indicating the level of secretion by the cartilage disk taken from that location, on a logarithmic scale.

Statistical Analysis

All data are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using Systat 10.2 software. ***Experiment I.*** PRG4 secretion values were log transformed for normality, and effects of joint region (PFG, LFC, MFC) and site (indicated by dots, Fig. 3B) on PRG4 secretion by cartilage explants from freshly harvested joints were determined by repeated measures ANOVA (region and site as repeated factors) and post-hoc comparisons for the interaction between region and site. ***Experiment II.*** *PRG4 Expression:* Percentage data were arcsine transformed, and effects of CPM and duty cycle were determined by ANOVA with Tukey post-hoc tests. *PRG4 Secretion:* Secretion values were log transformed and effects of CPM and joint region were determined by repeated measures ANOVA.

RESULTS

Experiment I (Fig. 2): Fresh Joints

Intrinsic chondrocyte PRG4 secretion, determined from freshly isolated joints, was highly dependent on cartilage location (Fig. 3B). PRG4 secretion from cartilage disks varied markedly with explant site (indicated by dots, Fig. 3) within the LFC ($p<0.01$) and MFC ($p<0.001$) regions, but not within the PFG region ($p=0.78$). Averaging secretion values over all sites

within a given region suggested (p=0.1) that in general, cartilage from the PFG and MFC secreted similar amounts of PRG4 on average (55±18, 42±7 μg/(cm^2*day), respectively), while cartilage from the LFC secreted only 27±5 μg/(cm^2*day). Finally, PRG4 secretion rates varied strikingly among the 4 animals, with the coefficient of variation (CV) of PRG4 secretion values at a given cartilage explant site ranging from 13 to 158%.

Experiment II (Fig. 2): Bioreactor-Cultured Joints

CPM stimulation up-regulated PRG4 biosynthesis in cartilage from certain locations of the joint. PRG4 immunolocalization (Figs. 4A, 5) revealed that the percent of the chondrocyte population expressing PRG4 was regulated by CPM in a sliding duty cycle dependent manner (p<0.001 for interaction between CPM and sliding duty cycle). The regions of the femoral condyles that were continuously sliding against cartilage/meniscus in the joint cultured with CPM (Figs. 5I,L) contained more PRG4+ chondrocytes (LFC: 37±4%, p<0.001; MFC: 40±5%, p<0.01), compared to the same regions of the joint cultured without CPM (Figs. 5B,E), where 13±3% and 22±2% of chondrocytes expressed PRG4, respectively, in the LFC and MFC. In addition, cartilage from the region of the LFC that was intermittently sliding against cartilage/meniscus (Figs. 5C,J) had higher PRG4 expression (p<0.01) with CPM (36±8%) than without (13±4%), though an effect of CPM was not detected for the intermittently sliding region of the MFC (p=0.99, Figs. 5F,M), where 37±5% of chondrocytes were PRG4+. As expected, PRG4 expression was independent of CPM for the PFG region (52±2%, p=0.99, Figs. 5A,H) and for regions of the LFC (43±1%k, p=1.0) and MFC (40±5%, p=1.0) that were never sliding against cartilage/meniscus during CPM (Figs. 5D,K,G,N).

Bioreactor culture with CPM appeared to also maintain chondrocyte PRG4 secretion in a sliding duty-cycle dependent manner (duty-cycle regions shown in Fig. 3A), compared to bioreactor culture without joint motion (Figs. 3C, 4B). Consistent with effects on PRG4 expression, PRG4 secretion in continuously sliding regions of the LFC tended to be higher (p=0.2) in cartilage from the CPM stimulated joint (11±5 μg/(cm^2*day)) than in cartilage from the same region of the joint cultured without CPM (4±2 μg/(cm^2*day)). Averaged over all sites in a given region, PRG4 secretion also varied with joint region (p<0.05), consistent with the trend for cartilage from freshly isolated joints (Experiment I). The PFG and MFC secreted more PRG4 (40±7 and 27±8 μg/(cm^2*day), respectively, p<0.05 each) than cartilage from the LFC (10±2 μg/(cm^2*day)). Cartilage from the PFG regions of joints cultured both with CPM and without CPM secreted similar amounts of PRG4 (p>0.9). As expected, CPM stimulation did not affect PRG4 secretion by cartilage from sub-regions of the LFC (12±5 μg/(cm^2*day), p=1.0) and MFC (12±4 μg/(cm^2*day), p=0.56) that were never sliding against cartilage/meniscus during CPM stimulation. Secretion values were also unaffected by CPM in regions of intermittent sliding (LFC: 14±5 μg/(cm^2*day); MFC: 27±10 μg/(cm^2*day), p>0.9 each).

DISCUSSION

These results identify a mechanism by which joint motion and CPM may contribute to maintenance of joint health, and demonstrate the ability to culture whole joints in a bioreactor. CPM increased the percentage of chondrocytes expressing PRG4 (Figs. 4A, 5B,E,I,L) and tended to increase PRG4 secretion (Figs. 3C, 4B) for cartilage in the regions of the femoral condyles that were continuously sliding against adjacent meniscus/cartilage, compared to those values for the corresponding regions of joints cultured without CPM. Intermittent sliding against adjacent meniscus/cartilage was also sufficient to up-regulate chondrocyte PRG4 expression in cartilage from the LFC (Figs. 4A, 5C,J). Chondrocyte viability remained high during bioreactor culture of joints and was independent of applied continuous passive motion, suggesting that differences in PRG4 metabolism were due to mechanical regulation in certain

regions, rather than cell death. In addition, the marked site-associated variation in intrinsic chondrocyte PRG4 secretion over the joint surface was revealed (Fig. 3B).

The parameters of rehabilitative joint motion, applied through CPM, were chosen based on previous protocols found to be stimulatory, and also consideration of knee joint kinematics. For example, the cycling frequency (43 seconds per cycle) is similar to that found most effective in healing cartilage defects in rabbits[28], and is in the range of frequencies typically used for *in vitro* experimentation with CPM (40–45 seconds per cycle[21, 29, 30]). The range of motion used here was within the range of flexion angles typically experienced during walking (0–41° minimum to 50–77° maximum flexion angle) for both human knee joints[31, 32] and ovine stifle joints[33]. This study did not attempt to reproduce joint muscle forces, which also contribute to normal active and passive loading of the joint (total joint compressive forces up to 3 times body weight[34]). Axial loading in the present experiments was due to forces in the intact cruciate and collateral ligaments[35]. Thus the loading applied here is more similar in nature to that applied during CPM rehabilitative therapies than to that experienced during daily activities. The study of physiological levels of axial compression in addition to the CPM stimulation protocol could provide additional insight into the biomechanical regulation of chondrocyte metabolism *in vivo*.

Also, since the goal of this study was to determine, in a controlled manner, the effects of continuous passive motion on chondrocyte metabolism, several joint tissues were removed. While patellofemoral joint forces contribute to the kinematics of knee motion *in vivo*[36–38], removal of the patella allowed for an internal control region (i.e. PFG) of cartilage that experienced the same mechanical environment in joints cultured both with and without CPM. Synovial fluid was also removed, and the medium used to bathe the joint was a baseline formulation typically used for cartilage and chondrocyte cultures, different in composition from the synovial fluid that normally bathes cartilage *in vivo*. This created a well-defined biochemical environment that allowed effects of CPM itself to be determined. Fetal bovine serum was added during bioreactor culture, as it has been shown to maintain cartilage proteoglycan synthesis *in vitro*[39]. Specific chemokines and growth factors could also be added during bioreactor culture, without CPM to assess the effects of biochemical stimuli on chondrocytes in intact cartilage, or with CPM to investigate possible interactive effects of biomechanical and biochemical stimuli.

While many factors are important to the maintenance of joint health, PRG4 metabolism was chosen for study because it is a functional lubricant whose expression is mechanosensitive. First, since PRG4 molecules function to provide boundary lubrication during cartilage-on-cartilage sliding[7], it was hypothesized that mechanical conditions that require lubrication (cartilage sliding against cartilage and meniscus in a physiological configuration) might result in increased PRG4 production. Furthermore, chondrocyte PRG4 metabolism is markedly regulated by micro-environmental cues during short term culture, including both biochemical[40, 41] and biomechanical[15, 16] stimuli, such that effects of rehabilitative joint motion were likely to be detected. PRG4 expression (by immunolocalization) provided a snapshot of chondrocyte populations expressing PRG4 immediately (during the 4 hr monensin treatment period) upon termination of CPM, while PRG4 secretion provided a functional measure of lubricant production over 2 days following bioreactor culture. The difference in timing between these measures of PRG4 biosynthesis, as well as the difference in quantity being measured (cells expressing PRG4 versus amount of PRG4 secreted) could account for the differences between effects of loading on PRG4 secretion and on PRG4 expression, for example, in the case of the intermittently stimulated region of the LFC (Figs. 4AB and 5CJ). The immunolocalization analysis of PRG4 may be expected to be more sensitive to loading than the secretion analysis, since the effects of certain types of load on PRG4 secretion are diminished within the days following termination of applied load[15, 16]. The marked intrinsic

topographical variation in these measures may be due to the high mechano-sensitivity of chondrocyte expression of PRG4, combined with wide variations in biomechanical environments experienced by different joint regions. The high inter-animal variation could account for the slightly lower overall PRG4 secretion levels in the bioreactor cultured joints compared to the levels secreted by freshly harvested joints, which were from different animals. However, the use of animal-matched joints (right and left from the same animal) for each run of experiment II eliminated this issue for comparison of joints cultured in bioreactors with and without CPM. Alternatively, the lower overall PRG4 secretion from bioreactor cultured tissue could be due to the equilibration of the cartilage in culture medium for 24 hours (during bioreactor culture) prior to explant culture.

The novel bioreactor for CPM stimulation of whole joints developed in this study provided the means to investigate mechano-regulation of PRG4 metabolism in a loading environment that is more physiological than that of traditional bioreactor culture systems, while at the same time eliminating some of the confounding factors present with *in vivo* studies. The marked site-associated variation in chondrocyte PRG4 secretion and expression and the apparent dependence on local mechanical environment, taken together with the altered PRG4 expression patterns seen in tibial cartilage subjected to abnormal mechanical stimulation[12], further support the hypothesis that mechanical factors are key determinants of PRG4 metabolism *in vivo*. CPM stimulation resulted in up-regulation of PRG4 secretion and expression levels by nearly 3-fold in the continuously stimulated region of the LFC, consistent with the 3-fold increase in proteoglycan production resulting from tissue shear of cylindrical cartilage explants[16], and the 3 to 7-fold up-regulation of PRG4 mRNA due to surface motion applied to chondrocytes in cartilaginous constructs[13]. These results also raise the possibility that regulation of PRG4 metabolism may depend on loading duty cycle, as intermittent cartilage sliding was sufficient to alter PRG4 expression in the LFC, though this did not occur in the MFC. Since intermittent sliding did not appear sufficient to alter chondrocyte secretion of PRG4 in either femoral condyle, it may be that intermittent sliding led to transient up-regulation, whereas continuous sliding resulted in longer-lasting effects.

The finding that CPM during culture maintains chondrocyte viability and metabolic function supports the use of CPM, instead of immobilization, as a post-operative treatment, and may indicate a mechanism by which CPM is beneficial to the health of cartilage and joints *in vivo*. In addition, PRG4 synthesis was very low in cartilage from certain regions of the joint cultured without CPM, consistent with previous reports that joint immobilization causes decreased chondrocyte metabolism of matrix molecules, which results in decreased cartilage thickness and mechanical integrity[42–45]. Thus, these results suggest that the negative effects of immobilization may also be due to down-regulation of chondrocyte synthesis of lubricant and chondroprotective PRG4 molecules.

The higher up-regulation of PRG4 secretion and expression in the LFC (3-fold for both measures) compared to the MFC (1.5-fold for secretion, ~1.8-fold for expression) in the continuously sliding region, as well as the ability of intermittent sliding to up-regulate PRG4 expression in the LFC but not the MFC, could be due to differences in biomechanical environment experienced by the two regions during CPM. In humans, the LFC and MFC experience distinct motion patterns relative to the adjacent tissues during knee flexion, with the MFC tending to move by sliding against the tibial plateau, while the LFC tends to "roll" across the tibial plateau[46–48]. This may also be the case for the bovine stifle joint, although with anatomically opposite tendencies (LFC sliding, MFC rolling), as the relative size and shape of the bovine femoral condyles and patellofemoral groove is opposite to that of humans. These observations suggest that sliding, which would impart shearing deformation to the tissue, leads to more up-regulation of PRG4 secretion than rolling, which would sequentially impart compressive deformation to different sites. Alternatively, the up-regulation of PRG4

expression and secretion in certain regions could be due to increased nutrient transport to those regions resulting from motion of the joint (compared to the transport level during bioreactor culture without motion), the unique joint loading patterns applied during CPM, or regional differences in cell phenotype.

The CPM bioreactor for whole joints developed in this study provides a platform for possible future *in vitro* studies and applications, including the assessment of the effects of CPM on metabolism of various joint tissues and possible interactions of biomechanical and biochemical signals. This system could also be used to investigate the effects of rehabilitative joint motion on the efficacy of various cartilage defect repair strategies in live tissue *in vitro*, as described for post mortem tissue[49], before moving on to more costly and complicated *in vivo* studies[50]. The current system allows for the simultaneous culture of two joints, but could be scaled up for culture of many joints in parallel. In addition, his bioreactor may be useful for *in vitro* culture of biological joints which could ultimately be used as restorative implants for large cartilage defects, and possibly whole joints.

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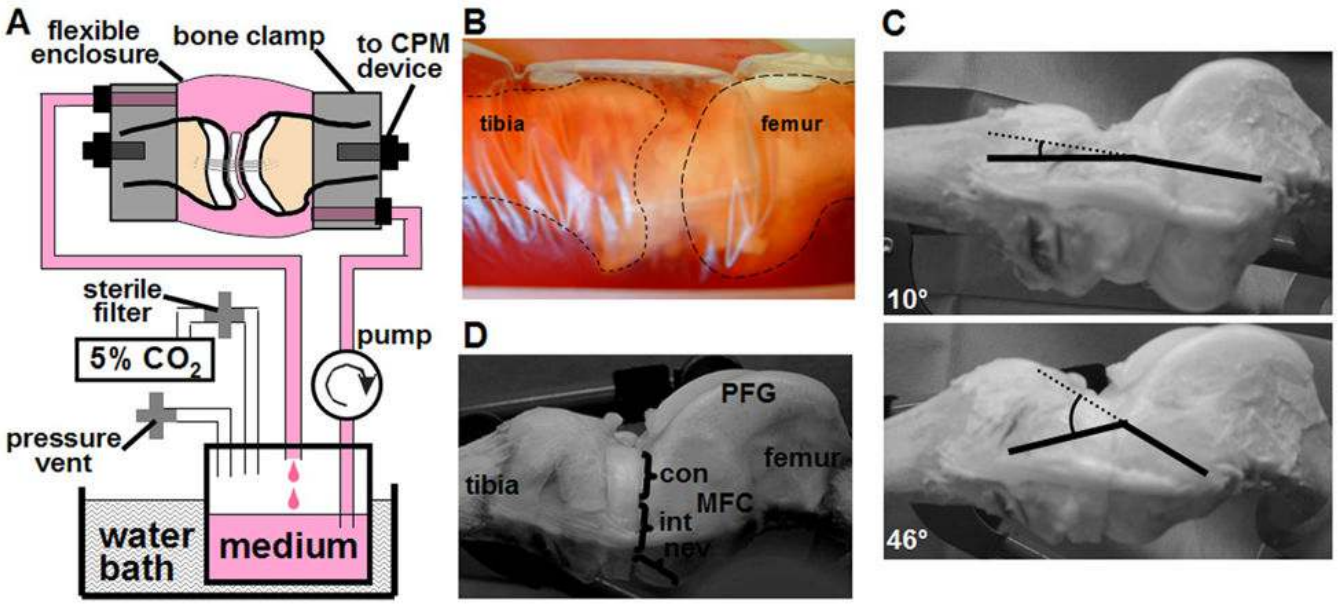

Figure 1.

Continuous passive motion bioreactor system for whole joints. (**A**) Schematic of system for maintaining tissue-culture conditions during continuous passive motion stimulation of the joint. (**B**) Actual joint in bioreactor. (**C**) During motion stimulation, the joint oscillates between 10° and 46° of flexion, such that certain regions of the femoral condyles (**D**) are continuously (con), intermittently (int), or never (nev) sliding against opposing tissues during stimulation.

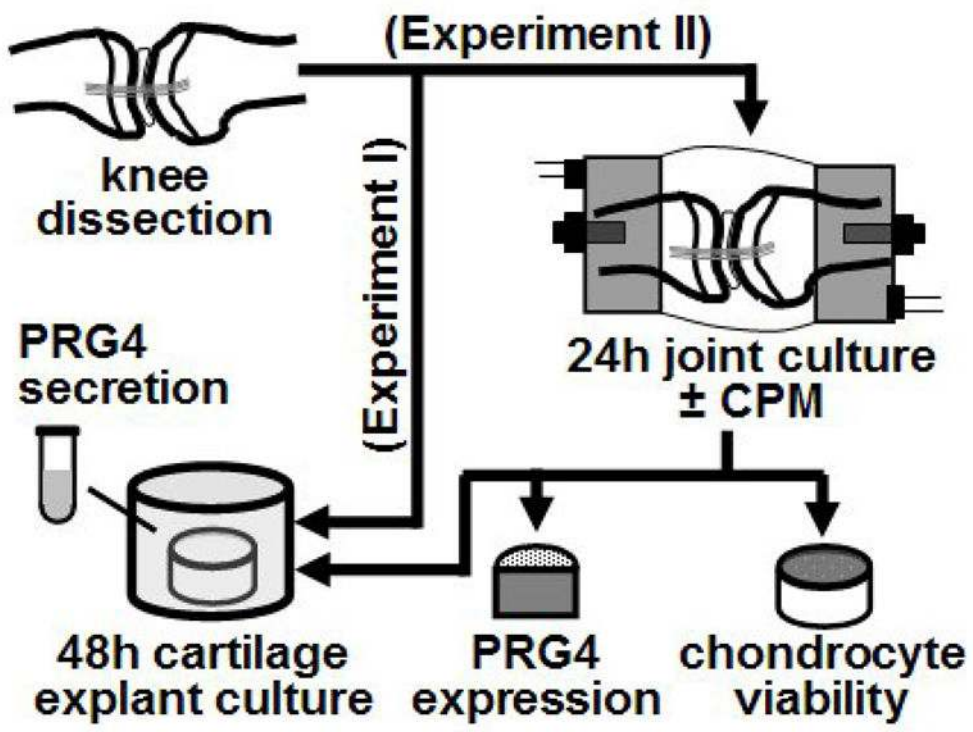

Figure 2.
Bovine stifle joints were isolated, dissected, and used in one of two separate experiments. (Experiment I) Cylindrical cartilage disks were explanted and cultured for 2 days. (Experiment II) Joints were cultured for 24 hours in the bioreactor with or without CPM stimulation, and cartilage disks were then explanted and analyzed for chondrocyte viability or chondrocyte PRG4 expression, or cultured for 2 days. PRG4 secreted by cartilage explants during culture was quantified and characterized from the conditioned medium samples.

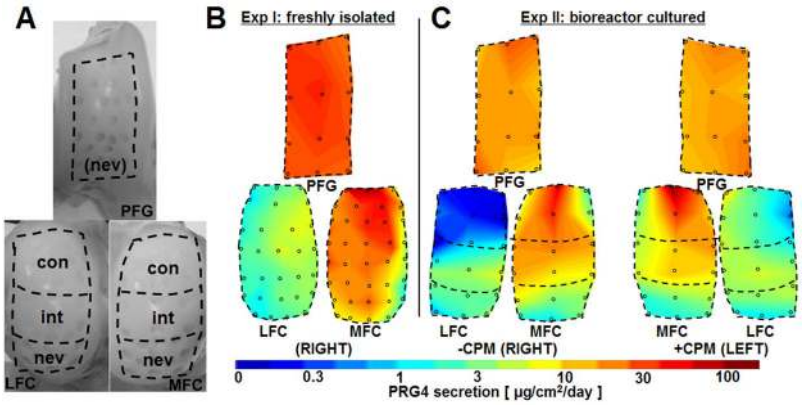

Figure 3.

(**A**) Cartilage disks were explanted from various sites on the patellofemoral groove (PFG) and lateral and medial femoral condyles (LFC, MFC), and grouped into sub-regions that continuously (con), intermittently (int), or never (nev) slide against opposing tissues during CPM stimulation. PRG4 secretion by cartilage disks from freshly harvested (**B**, Experiment I) or bioreactor cultured (**C**, Experiment II, with or without CPM stimulation) joints, represented as a logarithmic color scale contour mapping of mean secretion values onto the joint regions. Explant sites are shown as open dots.

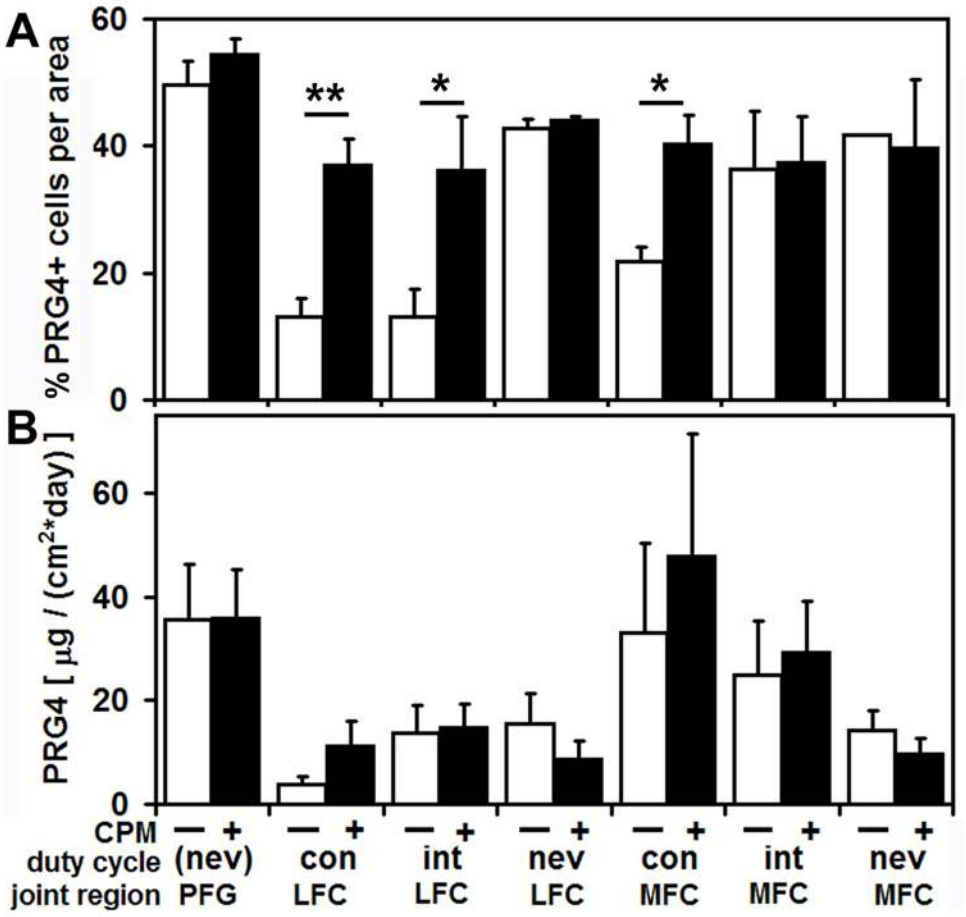

Figure 4.

(**A**) Chondrocyte PRG4 expression in cartilage sections, and (**B**) PRG4 secretion of cartilage disks from the patellofemoral groove (PFG) and various sub-regions (based on sliding duty cycle) of the lateral and medial femoral condyles (LFC, MFC) of bovine stifle joints after culture in a whole joint bioreactor with (■) or without (□) CPM stimulation. Mean±SEM. n=3–4 animals. *p<0.01, **p<0.001.

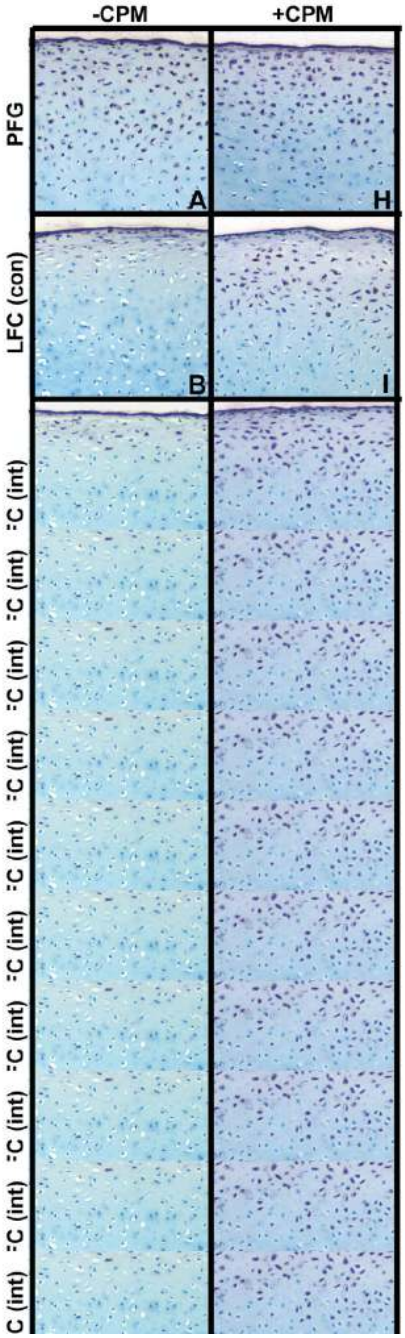

Figure 5.
Immunolocalization of PRG4 (dark purple stain) within chondrocytes. Shown are representative images of cartilage samples from various joint regions, after bioreactor culture without (−**CPM, A–G**), or with CPM stimulation (**+CPM, H–N**). Bar = 100 μm.