Research Article

Phosphocitrate Is Potentially a Disease-Modifying Drug for Noncrystal-Associated Osteoarthritis

Yubo Sun,1 David R. Mauerhan,1 Atiya M. Franklin,1 James Norton,2 Edward N. Hanley Jr.,1 and Helen E. Gruber1

1 Department of Orthopedic Surgery, Carolinas Medical Center, P.O. Box 32861, Charlotte, NC 28232, USA
2 Department of Biostatistics, Carolinas Medical Center, P.O. Box 32861, Charlotte, NC 28232, USA

Correspondence should be addressed to Yubo Sun; yubo.sun@carolinas.org

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Phosphocitrate (PC), a calcification inhibitor, inhibits the development of crystal-associated osteoarthritis (OA) in Hartley guinea pigs. However, the molecular mechanisms underlying its disease-modifying effect remain elusive. This study sought to test the hypothesis that PC has calcium crystal-independent biological activities which are, at least in part, responsible for its disease-modifying activity. We found that PC inhibited the proliferation of OA fibroblast-like synoviocytes in the absence of calcium crystals. Consistent with its effect on cell proliferation, PC downregulated the expression of numerous genes classified in cell proliferation. PC also downregulated the expression of many genes classified in angiogenesis and inflammatory response including prostaglandin-endoperoxide synthase 2, interleukin-1 receptor, type I, and chemokine (C-C motif) ligand 2. In contrast, PC upregulated the expression of many genes classified in musculoskeletal tissue development, including aggrecan, type I collagen, and insulin-like growth factor binding protein 5. These findings suggest that PC is not only a promising disease-modifying drug for crystal-associated OA but also for noncrystal-associated OA.

1. Introduction

Osteoarthritis (OA) is a progressive disorder characterized by the degeneration of articular cartilage, formation of osteophytes, and synovial lining hyperplasia. It is one of the most prevalent causes of disability in the aging population and has enormous economic and social consequences. However, existing treatment options only provide symptomatic relief and have no effect on the progression of the underlying disease. The lack of progress in the development of disease-modifying drugs for OA therapy is largely due to our limited understanding of the pathogenesis of the disease and our insufficient knowledge about the molecular targets for intervention.

The most apparent pathological changes in OA are usually found in articular cartilage. Synovium of patients with OA has traditionally been considered to be normal and is used as control in studies to investigate the pathological changes in the synovium of patients with rheumatoid arthritis (RA) [1, 2]. OA fibroblast-like synoviocytes (FLSs) have also been used as control cells in some studies [3, 4]. However, synovial lining hyperplasia and inflammation, a potential leading cause of knee pain, are common findings in OA patients [5, 6]. Studies have demonstrated that OA synovium and OA FLSs display different gene expression profiles compared with the synovium and FLSs derived from normal control subjects [7, 8]. These findings indicate that OA synovium plays an important role in the pathogenesis of OA.

Basic calcium phosphate (BCP) crystal and calcium pyrophosphate dihydrate (CPPD) crystal are the two most common intra-articular crystals [9, 10]. The presence of these crystals in the synovial fluid or joint tissues of end-stage OA patients is a well-recognized event [11–14]. In cell culture, calcium crystals induced mitogenesis, production of matrix metalloproteinases (MMPs), and endocytotic activity of cells [15–18]. However, the clinical significance of these calcium crystals and their role in the development and/or progression of OA remain unclear [14, 19–21].
Phosphocitrate (PC), a potent anticalcification molecule, was originally identified in mammalian mitochondria [22]. PC inhibits the formation and growth of calcium crystals by its strong binding to amorphous calcium phosphates aggregates and the surface of calcium crystals. In cell cultures, PC inhibited crystal-induced mitogenesis, expression of MMPs, and cell death [23–25]. In Hartley guinea pig model of crystal-associated OA, PC inhibited meniscal calcification and reduced the degeneration of articular cartilage [26]. These findings appear to provide support for the notions that calcium crystals play an important role in the development and/or progression of OA and that calcification inhibitors are promising disease-modifying drugs for crystal-associated OA therapy [27]. However, a bisphosphonate, a potent calcification inhibitor similar to PC, failed to inhibit the development of OA in Hartley guinea pigs, raising questions about the role of crystals in the development of crystal-associated OA [28]. Several studies indicate that PC has biological activities unrelated to its anticalcification activity. For example, PC reduced the degradation of low-density lipoprotein by 60% [29]. PC inhibited transforming growth factor-β-induced proliferation of progressive ankylosis fibroblasts [30].

We decided to perform this study to test the hypothesis that PC has unique crystal-independent biological activities which are responsible, at least in part, for its disease-modifying activity.

2. Materials and Methods

Dulbecco’s modified eagle medium (DMEM), fetal bovine serum (FBS), and stock antibiotic/antimycotic mixture were products of Invitrogen (Carlsbad, CA, USA). PC was synthesized according to the procedure previously described [31].

2.1. Cell Cultures. Telomerase-transduced human OA FLSs, or hTERT-OA 13A FLSs, have been previously described [8]. Primary OA FLSs were prepared from synovial tissues collected with the approval of the authors’ institutional review board from OA patients undergoing total joint replacement surgery at our medical center. The need for informed consent was waived because the synovial tissues were surgical waste, and no private patient information was collected. Briefly, synovial tissues were minced into small pieces (3 mm × 3 mm) and cultured in 100 mm plates at 37°C in DMEM containing 10% FBS. Every three days, culture medium was changed. When OA FLSs reached 80% confluence, they were harvested and passaged. Human foreskin fibroblasts were obtained from ATCC (CRL-2429, Mananas, USA) and expanded in DMEM containing 10% FBS.

2.1.1. Cell Proliferation. hTERT-OA 13A FLSs (4 × 10⁴) were plated in six-well cluster plates. On the second day, DMEM containing 10% FBS and PC was added to the top three wells. DMEM containing 10% FBS without PC was added to bottom three wells. The culture medium was changed every three days until the cells in the bottom wells without PC reached 85% confluence. All cells were then harvested and cell numbers were determined by cell count using a hemocytometer. This proliferation assay was also performed using primary OA FLSs and foreskin fibroblasts for comparison.

2.2. RNA Extraction. hTERT-OA 13A FLSs were plated in four 100 mm plates at 90% confluence. On the second day, DMEM containing 1% FBS was added. On the third day, DMEM in two plates was replaced with DMEM containing 1% FBS and PC at a final concentration of 0.6 mM. DMEM in other two plates was replaced with DMEM containing 1% FBS but without PC. Twenty-four hours later, total RNA was extracted from these cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and purified using Oligotex kit (Qiagen, Valencia, CA, USA). These RNA samples were used for microarray analyses and RT-PCR experiments.

2.3. Microarray. Double-stranded DNA was synthesized using SuperScript double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). The DNA product was purified using GeneChip sample cleanup module (Affymetrix, Santa Clara, CA, USA). cRNA was synthesized and biotin labeled using BioArray high yield RNA transcript labeling kit (Enzo Life Sciences, Farmingdale, NY, USA). The cRNA product was purified using GeneChip sample cleanup module and subsequently chemically fragmented. The fragmented, biotinylated cRNA was hybridized to HG-U133_Plus_2 gene chip using Affymetrix Fluidics Station 400 (Affymetrix, Santa Clara, CA, USA). The fluorescent signals were quantified during two scans by Agilent Gene Array Scanner G2500A (Agilent Technologies, Palo Alto, CA, USA) and GeneChip operating Software (Affymetrix, Santa Clara, CA, USA). Genesifter software (VizX Labs, Seattle, WA, USA) was used for the analysis of differential gene expression and gene ontology.

2.4. Real-Time RT-PCR. Briefly, cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems, Inc., University Park, IL, USA) using the RNA samples described. Quantification of relative transcript levels of selected genes was performed using ABI7000 Real-Time PCR system (Applied Biosystems, Inc., University Park, IL, USA). TaqMan Gene Expression assays (Applied Biosystems, Inc., University Park, IL, USA) were used. cDNA samples were amplified with an initial Taq DNA polymerase activation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for one minute. Fold change was calculated, and the expression level of the genes was normalized to the expression level of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) according to the method described [32]. The experiments were performed independently using RNA samples extracted from both hTERT-OA 13A FLSs and primary OA FLSs.

2.5. Statistical Analysis. Data are expressed as the mean ± SD. For cell proliferation, the difference between two experimental groups was analyzed using Student’s t-test. For real-time RT-PCR, experiment was repeated twice in triplicates. The difference between two experimental groups was analyzed using Student’s t-test.
Table I: Differentially expressed genes in PC-treated hTERT-OA13AFLS compared with the untreated cells.

| Biological process | Gene name      | Gene ID      | Differ. expr. (fold)* | Description                                                  |
|--------------------|----------------|--------------|------------------------|--------------------------------------------------------------|
|                    | BLM            | NM_000057    | -3.64                  | Bloom syndrome                                               |
|                    | CCNE2          | AF112857     | -3.74                  | Cyclin E2                                                   |
|                    | CCNE1          | A1671049     | -2.30                  | Cyclin E1                                                   |
|                    | CDC25A         | AY137580     | -3.63                  | Cell division cycle 25 homolog A (S. pombe)                 |
|                    | CDC25C         | NM_001790    | -2.31                  | Cell division cycle 25 homolog C (S. pombe)                 |
| Cell proliferation | CDC2           | AA749427     | -3.13                  | Cell division cycle 2, G1 to S and G2 to M                 |
|                    | CDC6           | NM_001254    | -2.36                  | Cell division cycle 6 homolog (S. cerevisiae)               |
|                    | CDC7           | NM_003503    | -2.12                  | Cell division cycle 7 homolog (S. cerevisiae)               |
|                    | CDCA5          | BE614410     | -2.41                  | Cell division cycle associated 5                           |
|                    | CDCA8          | BC001651     | -2.11                  | Cell division cycle associated 8                           |
|                    | CDK2           | AB012305     | -2.74                  | Cyclin-dependent kinase 2                                   |
|                    | NCAPH          | D38553       | -2.64                  | Non-SMC condensin I complex, subunit H                       |
|                    | HELLS          | NM_018063    | -2.49                  | Helicase, lymphoid specific                                 |
|                    | AURKB          | AB011446     | -2.43                  | Aurora kinase B                                             |
|                    | KIF23          | AW192521     | -2.41                  | Kinesin family member 23                                    |
|                    | CLASP2         | BC029035     | -2.40                  | Cytoplasmic linker-associated protein 2                      |
|                    | NUF2           | AF326731     | -2.35                  | NUF2, NDC80 kinetochore complex component, homolog           |
|                    | DSN1           | NM_024918    | -2.35                  | DSN1, MIND kinetochore complex component, homolog            |
|                    | SPC24          | AI469788     | -2.32                  | SPC24, NDC80 kinetochore complex component, homolog          |
|                    | SPC25          | AF225416     | -2.10                  | SPC25, NDC80 kinetochore complex component, homolog          |
|                    | HMGA2          | A1990940     | -2.30                  | High mobility group AT-hook 2                               |
|                    | LIG1           | NM_000234    | -2.25                  | Ligase I, DNA, ATP-dependent                                 |
|                    | KIFC1          | BC000712     | -2.21                  | Kinesin family member C1                                    |
|                    | BRCA2          | X95152       | -2.18                  | Breast cancer 2, early onset                                 |
|                    | ERCC6L         | NM_017669    | -2.17                  | Excision repair cross-complement repair deficiency, comp. group 6 like |
|                    | SPAG5          | NM_006461    | -2.16                  | Sperm-associated antigen 5                                  |
|                    | NEK2           | ZZ5425       | -2.14                  | Never-in-mitosis-gene-A- (NIMA) related kinase 2             |
|                    | NCAPG          | NM_022346    | -2.12                  | Non-SMC condensin I complex, subunit G                       |
|                    | ZWINT          | NM_007057    | -2.01                  | ZW10 interactor antisense                                    |
|                    | PARD3B         | AF428251     | 3.24                   | Par-3 partitioning defective 3 homolog B (C. elegans)       |
|                    | 11-Sep         | A1333326     | 2.28                   | Septin II                                                   |
| Angiogenesis       | NRPI           | AF280547     | -2.69                  | Neuropilin 1                                                |
|                    | TEK            | BF594294     | -2.58                  | TEK tyrosine kinase, endothelial                            |
|                    | ELK3           | NM_005230    | -2.42                  | ELK3, ETS-domain protein (SRF accessory protein 2)          |
|                    | EREG           | NM_001432    | -1.90                  | Epiregulin                                                  |
|                    | PML            | AW291023     | -1.89                  | Promyelocytic leukemia                                      |
|                    | COL15A1        | NM_001855    | -1.80                  | Collagen, type XV, alpha-1                                  |
|                    | NRP2           | A1819729     | -1.75                  | Neuropilin 2                                                |
|                    | SPHK1          | NM_021972    | -1.72                  | Sphingosine kinase 1                                         |
|                    | FOXC2          | NM_005251    | -1.68                  | Forkhead box C2, mesenchyme forhead 1 (MFH-1)               |
|                    | SCG2           | NM_003469    | -1.66                  | Secretogranin II (chromogranin C)                           |
|                    | EDNRA          | NM_001957    | -1.56                  | Endothelin receptor type A                                   |
| Biological process          | Gene name | Gene ID | Differ. expr. (fold)* | Description                                      |
|-----------------------------|-----------|---------|----------------------|--------------------------------------------------|
| **Inflammatory response**   | PTGS2     | AY151286 | −6.09                | Prostaglandin-endoperoxide synthase 2             |
|                            | SERPINA1  | AF19873  | −2.15                | Serpin peptidase inhibitor, clade A              |
|                            | GPR68     | A1805006 | −2.15                | G protein-coupled receptor 68                    |
|                            | BMPR1B    | A935461  | −2.12                | Bone morphogenetic protein receptor, type IB     |
|                            | EVII      | BE466525 | −2.00                | Ecotropic viral integration site 1               |
|                            | FOS       | BC004490 | −1.92                | V-fos FB/J murine osteosarcoma viral oncogene homolog |
|                            | IRAK2     | AI246590 | −1.82                | Interleukin-1 receptor-associated kinase 2       |
|                            | CCL2      | S69738   | −1.82                | Chemokine (C-C motif) ligand 2                   |
|                            | CCR1      | NM_001295| −1.60                | Chemokine (C-C motif) receptor 1                 |
|                            | CXCL2     | M57731   | −1.66                | Chemokine (C-X-C motif) ligand 2                 |
|                            | SPN       | BC035510 | −1.79                | Sialophorin (leukosialin, CD43)                  |
|                            | TLR4      | AFI77765 | −1.70                | Toll-like receptor 4                             |
|                            | SCG2      | NM_003469| −1.66                | Secretogranin II (chromogranin C)               |
|                            | FNI       | AF276395 | −1.58                | Fibronectin 1                                   |
|                            | KLKB1     | BE326857 | −1.52                | Cytochrome P450, family 4, subfamily V, polypeptide 2 |
|                            | NDST1     | NM_001543| 2.05                 | N-Deacetylase/N-sulfotransferase (heparan glucosaminyl) 1 |
|                            | C3        | NM_000064| 2.05                 | Complement component 3                          |
|                            | SERPINA3  | NM_001085| 1.88                 | Serpin peptidase inhibitor, clade A              |
|                            | SBTN2O2   | AC005390 | 1.78                 | Strawberry notch homolog 2 (Drosophila)          |
|                            | NFKB1Z    | BE646573 | 1.74                 | Nuclear factor of kappa light polypeptide enhancer in B-cells inhibitor zeta |
|                            | MASPI     | NM_001879| 1.64                 | Mannan-binding lectin serine peptidase 1        |
|                            | STAT3B    | NM_012448| 1.59                 | Signal transducer and activator of transcription 5B |
| **Response to pain**        | TACR1     | AA461490 | −4.39                | Tachykinin receptor 1                            |
|                            | COMT      | BG149428 | −2.03                | Catechol-O-methyltransferase                     |
|                            | CACNA1A   | BC042451 | −2.00                | Calcium channel, voltage-dependent, P/Q type, alpha-1A subunit |
| **Response to interleukin-1**| SRC       | NM_005417| −3.95                | V-src sarcoma viral oncogene homolog (avian)     |
|                            | IRAK2     | AI246590 | −1.82                | Interleukin-1 receptor-associated kinase 2       |
|                            | PCSK1     | NM_000439| −1.60                | Proprotein convertase subtilisin/kexin type 1    |
|                            | IL1R1     | AK026803 | −1.59                | Interleukin-1 receptor, type I                   |
|                            | GHR       | NM_000163| 1.76                 | Growth hormone receptor                          |
| **Cytokine- and chemokine-mediated** | EREG     | NM_001432| −1.90                | Epiregulin                                       |
|                            | CCL2      | S69738   | −1.82                | Chemokine (C-C motif) ligand 2                   |
|                            | LIFR      | NM_002310| −1.77                | Leukemia inhibitory factor receptor-alpha        |
|                            | RQCD1     | BC007102 | −1.76                | RCD1 required for cell differentiation homolog (S. pombe) |
|                            | LRP8      | NM_004631| −1.73                | Low-density lipoprotein receptor-related protein 8, apolipoprotein e receptor |
|                            | CCR1      | NM_001295| −1.60                | Chemokine (C-C motif) receptor 1                 |
|                            | IL1R1     | AK026803 | −1.59                | Interleukin-1 receptor, type I                   |
|                            | STAT3     | BF508977 | 2.01                 | Signal transducer and activator of transcription 3 |
|                            | STAT3B    | NM_012448| 1.59                 | Signal transducer and activator of transcription 5B |
was analyzed using Student’s t-test. In all cases, $P$ values less than 0.01 were considered significant. Statistical analysis was performed using the SAS software, version 9.3.

### 3. Results

#### 3.1. PC Inhibited Proliferation of OA FLSs

PC had no effect on the proliferation of human foreskin fibroblasts (Figure 1(a), the left bar group). However, PC inhibited the proliferation of hTERT-OA 13A FLSs (Figure 1(a), the right bar group). There were about 60% fewer hTERT-OA 13A FLSs in the PC-treated wells after nine days of culture compared with the cells in the untreated wells ($P < 0.01$).

Next, we examined the effect of PC on cell proliferation using primary OA FLSs. For comparison, we also examined the effect of disodium salt of ethane-1-hydroxy-1,1-bisphosphonic acid (EHDP), which is a bisphosphonate. As shown in Figure 1(b), both PC and EHDP inhibited the proliferation of primary OA FLSs in a dose-dependent manner. The morphologies of PC-treated OA FLSs and untreated OA FLSs were similar (not shown), indicating that the reduction in cell number was not due to cellular toxicity of PC.

#### 3.2. Effect of PC on Gene Expression

Microarray analysis revealed that of the more than 50,000 transcripts examined, 3,011 transcripts displayed significant differential expression (more than 1.5-fold) between the PC-treated hTERT-OA 13A FLSs and the untreated hTERT-OA 13A FLSs; 1,558 transcripts were downregulated, and 1,453 transcripts were upregulated by PC. Differentially expressed genes were classified according to gene ontology category biological process. The genes that fell into specific biological processes previously implicated in OA, or suspected to have a role in OA, are listed in Tables 1 and 2.

As shown in Table 1, the expression of numerous genes classified in cell proliferation was downregulated by PC. Of the 32 differentially expressed genes with more than twofold changes between the PC-treated cells and untreated cells, the expression of 30 genes, including cyclin E1 (CCNE1; $-2.30$-fold change), cyclin E2 (CCNE2; $-3.74$-fold change), and cell division cycle 25 homolog C (CDC25C; $-2.31$-fold change), was downregulated by PC. This downregulatory effect of PC on the genes associated with cell proliferation is consistent with the finding that PC inhibited the proliferation of OA FLSs (Figure 1).

The expression of many genes classified in angiogenesis and inflammatory response was also downregulated by PC. Of the 18 differentially expressed genes classified in angiogenesis, the expression of 13 genes, including neuropilin 1 (NRP1; $-2.69$-fold change) and TEK tyrosine kinase (TEK; $-2.58$-fold change), was downregulated by PC. Of the 22 differentially expressed genes classified in inflammatory response, the expression of 15 genes, including prostaglandin-endoperoxide synthase 2/cyclooxygenase (PTGS2/Cox-2; $-6.09$-fold change), chemokine (C-C motif) ligand 2 (CCL2/MCP-1; $-1.82$-fold change), and chemokine (C-X-C) ligand 2 (CXCL2; $1.66$-fold change), was downregulated by PC. In addition, of the 3 differentially expressed genes classified in response to pain, the expression of all 3 genes, including tachykinin receptor 1 (TACR1, $-4.39$), was downregulated by PC. Of the 5 differentially expressed genes classified in the response to interleukin-1 (IL-1), the expression of 4 genes, including V-src sarcoma viral oncogene homolog (SRC; $-3.95$-fold change), IL-1 receptor type 1 (IL1R1; $-1.59$-fold change), and IL-1 receptor-associated kinase 2 (IRAK2; $-1.82$-fold change), was downregulated by PC.
Furthermore, the expression of many genes classified in the cytokine- and chemokine-mediated signal pathway and IL-6 production was downregulated by PC (Table 1). Of the 9 differentially expressed genes classified in the cytokine and chemokine-mediated signal pathway, the expression of 7 genes, including chemokine (C-C motif) receptor 1 (CCR1, −1.60-fold change), was downregulated by PC. Of the 7 differentially expressed genes classified in IL-6 production, the expression of 6 genes, including tumor necrosis factor, alpha-induced protein 3 (TNFAIP3, −2.07-fold), was downregulated by PC.

Most of the genes upregulated by PC fell into biological processes associated with musculoskeletal tissue development. For example, of the 25 differentially expressed genes classified in muscle tissue development, the expression of 15 genes, including insulin-like growth factor binding protein 5 (IGFBP5; 8.57-fold change), was upregulated by PC. Of the 28 differentially expressed genes classified in skeletal development, the expression of 18 genes, including annexin A2 (2.17-fold change), vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR; 2.11-fold change), aggrecan (ACAN; 1.80-fold change), collagen type 1, alpha-1 (COL1A1; 1.66-fold change), and collagen type XI alpha-1 (COL11A1; 1.50-fold change), was upregulated by PC (Table 2).

3.3. Real-Time RT-PCR. The genes selected for validation for the differential expression between the PC-treated and untreated hTERT-OA 13A FLSs by real-time RT-PCR were listed in Table 3. As shown, the differential expression of the genes examined were confirmed by real-time RT-PCR.

4. Discussion

Previous studies demonstrated that PC inhibited BCP crystal-stimulated mitogenesis and expression of PTGS2/Cox-2 [15, 33, 34]. These observations provided the bases for the hypothesis that PC is potentially a disease-modifying drug for crystal-associated OA. However, the findings presented in this study clearly indicate that the inhibitory activities of PC on mitogenesis and proliferation of OA FLSs and the downregulatory activity of PC on the expression of PTGS2/Cox-2 are intrinsic properties of PC. These distinct biological activities of PC are not dependent on the presence of calcium crystals.

Synovial hyperplasia is associated with the severity of knee pain and fast hyaline articular cartilage loss in OA [35, 36]. Synovial hyperplasia is also associated with synovial angiogenesis [37]. OA FLSs, but not normal control FLSs, induced a significant enhancement of angiogenesis [38]. These observations indicate that OA FLSs play an important role in the pathogenesis of OA. In this study, we demonstrated that PC not only downregulated the expression of numerous genes associated with cell proliferation but also downregulated the expression of many genes associated with angiogenesis. These findings indicate that PC is potentially an antisyovial hyperplasia agent.

PTGS2/Cox-2 is a molecular target for the management of arthritis pain and inflammation [39, 40]. The strong downregulation of PTGS2/Cox-2 by PC suggests that PC may have some effect on the pain associated with OA. This potential effect of PC on pain is further supported by the finding that PC significantly downregulated the expression of TACR1 (−4.39-fold change) and SRC (−3.95-fold change) (Table 1). TACR1 is a G protein-coupled receptor which is upregulated in the joint tissues of patients with painful OA [41]. SRC, a tyrosine kinase, plays a role in substance P signaling which has been implicated in many inflammatory diseases [42, 43]. These findings together indicate that PC is potentially an analgesic agent.

In this study, we demonstrated that PC strongly downregulated the expression of many genes classified in cytokine-
| Biological process      | Gene name | Gene ID | Differ. Expre. (fold)* | Description                                                |
|------------------------|-----------|---------|------------------------|------------------------------------------------------------|
| **Muscle tissue development** | IGFBP5   | AW157548| 8.57                   | Insulin-like growth factor binding protein 5               |
|                        | CACNB4    | NM_000726| 2.73                   | Calcium channel, voltage-dependent, beta-4-subunit           |
|                        | TPM1      | AI521618| 2.43                   | Tropomyosin 1 (alpha)                                       |
|                        | JAG1      | U61276  | 2.02                   | Jagged 1 (Alagille syndrome)                               |
|                        | MORF4L2   | H43976  | 1.90                   | Mortality factor 4 like 2                                  |
|                        | NRG1      | NM_013957| 1.88                   | Neuregulin 1                                               |
|                        | SIRT2     | BG722779| 1.86                   | Sirtuin (silent mating type information regulation 2 homolog) 2 |
|                        | NFI       | D12625  | 1.80                   | Neurofibromin 1                                            |
|                        | OBSL1     | BF446888| 1.78                   | Obscurin-like 1                                            |
|                        | MBLN1     | AA732240| 1.73                   | Muscleblind-like (Drosophila)                              |
|                        | TPM1      | NM_000366| 1.72                   | Tropomyosin 1 (alpha)                                      |
|                        | CAV2      | AA150110| 1.67                   | Caveolin 2                                                 |
|                        | RXRA      | BE675800| 1.66                   | Retinoid X receptor, alpha                                 |
|                        | NR2F2     | AI554245| 1.63                   | Nuclear receptor subfamily 2, group F, member 2             |
|                        | TCF7L2    | AV721430| 1.61                   | Transcription factor 7-like 2 (T-cell specific, HMG-box)    |
|                        | TBX2      | U28049  | -4.17                  | T-box 2                                                    |
|                        | ADRB2     | NM_000024| -2.36                  | Adrenergic, beta-2-receptor, surface                        |
|                        | SORT1     | BE742268| -1.93                  | Sortilin 1                                                 |
|                        | GJC1      | NM_005497| -1.77                  | Gap junction protein, gamma 1, 45 kDa                      |
|                        | CENPF     | U30872  | -1.77                  | Centromere protein F, 350/400 ka (mitosin)                  |
|                        | BCL2      | NM_000657| -1.71                  | B-cell CLL/lymphoma 2                                      |
|                        | TBX3      | U69556  | -1.71                  | T-box 3                                                    |
|                        | SDC1      | NM_002997| -1.65                  | Syndecan 1                                                 |
|                        | TBX5      | AW269421| -1.54                  | T-box 5                                                    |
|                        | RARB      | NM_015854| -1.51                  | Retinoic acid receptor, beta                                |
| **Skeletal development** | ANXA2     | D28364  | 2.17                   | Annexin A2                                                 |
|                        | VDR       | AA772285| 2.11                   | Vitamin D (1,25-dihydroxyvitamin D3) receptor              |
|                        | GNAS      | AI693143| 1.95                   | GNAS complex locus                                         |
|                        | ACAN      | NM_001135| 1.80                   | Aggrecan                                                   |
|                        | COL1A1    | AI743621| 1.66                   | Collagen, type I, alpha-1                                  |
|                        | COL1A2    | AA628535| 1.88                   | Collagen, type I, alpha-2                                  |
|                        | COL1A1    | NM_001854| 1.50                   | Collagen, type XI, alpha-1                                 |
|                        | COL1A2    | NM_001854| 1.50                   | Collagen, type XII, alpha-1                                |
|                        | MSX2      | D89377  | 1.85                   | Msh homeobox 2                                             |
|                        | GHR       | NM_000163| 1.76                   | Growth hormone receptor                                    |
|                        | MEF2C     | AL536517| 1.59                   | Myocyte enhancer factor 2C                                 |
|                        | THRA      | NM_003250| 1.57                   | Thyroid hormone receptor, alpha                            |
|                        | RUNX2     | AW469546| 1.55                   | Runt-related transcription factor 2                        |
|                        | CLEC3B    | NM_003278| 1.55                   | Exosome component 7                                         |
|                        | MEF2C     | N22468  | 1.55                   | Myocyte enhancer factor 2C                                 |
|                        | IGFBP4    | NM_001552| 1.54                   | Insulin-like growth factor binding protein 4                |
|                        | PRKRA     | AA279462| 1.53                   | Protein kinase, interferon-inducible RNA-dependent activator |
|                        | TNFRSF11B | NM_002546| 1.50                   | Tumor necrosis factor receptor superfamily, member 11b     |
Table 2: Continued.

| Biological process         | Gene name | Gene ID   | Differ. Expre. (fold)* | Description                                           |
|---------------------------|-----------|-----------|------------------------|-------------------------------------------------------|
|                            | BMPR1B    | AA935461  | −2.12                  | Bone morphogenetic protein receptor, type IB          |
|                            | ANKH      | AF274753  | −1.93                  | Ankylosis, progressive homolog (mouse)               |
|                            | ACVR2A    | NM_001616 | −1.89                  | Activin A receptor, type IIA                          |
|                            | CYTL1     | NM_018659 | −1.83                  | Cytokine-like 1                                      |
|                            | TBX3      | U69556    | −1.71                  | T-box 3 (ulnar mammary syndrome)                     |
|                            | SOX9      | NM_000346 | −1.71                  | SRY- (sex-determining-region-Y-) box 9               |
|                            | FOXC2     | NM_005251 | −1.68                  | Forkhead box C2 (MFH-1, mesenchyme forkhead 1)       |
|                            | KIAA1217  | BC017424  | −1.66                  | KIAA1217                                             |
|                            | MMP9      | NM_004994 | −1.61                  | Matrix metalloproteinase 9                           |
|                            | TGFBR2    | NM_003242 | −1.51                  | Transforming growth factor, beta-receptor II (70/80 kDa) |

Collagen biosynthetic process

| Gene name | Gene ID   | Differ. Expre. microarray | Differential expression RT-PCR* | Differential expression RT-PCR** |
|-----------|-----------|---------------------------|--------------------------------|---------------------------------|
| COL3A1    | AU146808  | 1.83                      | −3.64                          | −4.23                           |
| COLIA2    | AA628535  | 1.88                      | −2.49                          | −2.87                           |
| COLIA1    | AI743621  | 1.66                      | −3.74                          | −3.93                           |
| TRAM2     | AI986461  | 1.58                      | −2.31                          | −2.01                           |
| ITGA2     | N95414    | −1.74                     | −2.25                          | −2.45                           |

Collagen catabolic process

| Gene name | Gene ID   | Differ. Expre. microarray | Differential expression RT-PCR* | Differential expression RT-PCR** |
|-----------|-----------|---------------------------|--------------------------------|---------------------------------|
| MMP3      | NM_002422 | 3.18                      | −3.64                          | −4.23                           |
| ADAMTS5   | BI254089  | 3.32                      | −2.49                          | −2.87                           |
| ADAMTS14  | W60649    | 2.15                      | −3.74                          | −3.93                           |
| MMP9      | NM_004994 | −1.61                     | −2.31                          | −2.41                           |

*Negative number indicates decreased expression (fold) in PC-treated hTERT-OA 13A FLS compared with the untreated cells. Positive number indicates elevated expression (fold) in PC-treated hTERT-OA 13A FLS compared with the untreated cells.

Table 3: Differential expression confirmed by real-time RT-PCR.

| Gene name | Gene ID   | Differential expression microarray | Differential expression RT-PCR* | Differential expression RT-PCR** |
|-----------|-----------|-----------------------------------|--------------------------------|---------------------------------|
| BLM       | NM_000057 | −3.64                             | −4.23                          | −3.95                           |
|HELLS      | NM_018063 | −2.49                             | −2.87                          | −2.12                           |
|CCNE2      | AF112857  | −3.74                             | −3.93                          | −3.70                           |
|CDC25C     | NM_001790 | −2.31                             | −2.01                          | −1.94                           |
|EGRI       | AI459194  | −2.25                             | −2.45                          | −3.29                           |
|FOSL1      | BG251266  | −1.95                             | −2.31                          | −2.11                           |
|PLAUR      | U08839    | −1.92                             | −2.41                          | −3.22                           |
|PLA2G4A    | M68874    | −1.51                             | −1.86                          | −2.11                           |
|PTGS2      | AY151286  | −6.09                             | −5.77                          | −5.10                           |
|TACR1      | AA461490  | −4.39                             | −3.92                          | −3.45                           |
|CCL2       | S69738    | −1.82                             | −2.02                          | −1.79                           |
|GPR68      | AI805006  | −2.15                             | −2.54                          | −2.28                           |
|CACNA1A    | BC042451  | −2.00                             | −2.10                          | −2.22                           |
|IGFBP5     | AW157548  | 8.57                              | 6.32                           | 5.46                            |
|ADAMTS5    | BI254089  | 3.32                              | 2.81                           | 2.11                            |
|COLIA2     | AA628535  | 1.88                              | 1.40                           | 1.64                            |

* Differential expression—the numbers are the ratio of the relative expression level of a specific gene in PC-treated hTERT-OA 13A FLS compared with the relative expression level of the specific gene in the untreated hTERT-OA 13A FLSs. ** Differential expression—the numbers are the ratio of the relative expression level of a specific gene in PC-treated primary FLSs compared with the relative expression level of the specific gene in the untreated primary OA FLSs.
and chemokine-mediated signal pathway, including IL1RI, CCL2, and CCR1. IL-1β is a main inflammatory cytokine found within OA joints and represents one of the possible treatment targets. CCL2 is a small chemokine which recruits leukocytes to synovial inflammation site and is central to the development of pain and inflammation associated with OA [44]. The findings presented in this study indicate that PC may inhibit inflammatory cell activation and infiltration and that PC is potentially an anti-inflammatory agent.

We also demonstrated that PC downregulated the expression of plasminogen activator, urokinase (PLAU, −2.21-fold change), PLAU receptor (PLAUR, −1.92-fold change), early growth response 1 (EGR-1, −2.25-fold change), v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS, −1.65-fold change), and FOS-like antigen 1 (FOSL1, −1.87-fold change). PLAU is a serine protease which catalyzes the conversion of inactive zymogen plasminogen to active protease plasmin when binding to its receptor PLAUR. Plasmin is capable of degrading all the components of the extracellular matrix and activating other enzymes such as MMPs. Higher levels of PLAU and PLAUR were detected in arthritic specimens [45–47]. EGR-1, FOS, and FOSL1 are involved in FLSs activation and implicated in the pathogenesis of RA [48–50]. The downregulation of these genes by PC supports the hypothesis that PC is potentially a disease-modifying drug for OA therapy.

Interestingly, PC upregulated the expression of many genes classified in muscle and skeletal tissue development, including IGFBP5. Previous studies demonstrated that inhibition of IGFBP5 proteolysis improved the joint architecture and reduced articular cartilage loss in a dog model of OA [51–53]. The strong upregulation of IGFBP5 by PC is clearly consistent with the notion that PC is potentially a disease-modifying drug for OA therapy. Moreover, PC upregulated the expression of ACAN (1.80-fold change), COL1A1 (1.66-fold change), COL1A2 (1.88-fold change), COL3A1 (1.83-fold change), COL11A1 (1.50-fold change), and COL12A2 (1.93-fold change), indicating that PC may improve the integrity of synovial tissues.

Surprisingly, PC upregulated the expression of MMP3 (3.18-fold change) and ADAM metallopeptidase with thrombospondin type 1 motif 5 (ADAMTS5; 3.32-fold change) (Table 2). The upregulation of both extracellular matrix proteins and extracellular matrix protein-degrading enzymes by PC indicates that PC may promote synovial tissue repair and regeneration. The implication of this specific effect on the disease process of OA is unclear at present.

5. Conclusions

PC is potentially an anti-synovial hyperplasia, analgesic and anti-inflammatory agent. PC is not only a promising disease-modifying drug for crystal-associated OA but also a promising disease-modifying drug for non-crystal-associated OA. The findings presented in this study provide further support for the development of PC, and/or its analogues, as disease-modifying drugs for OA therapy.

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