Published in final edited form as:
Arthritis Rheum. 2011 June ; 63(6): 1613–1625. doi:10.1002/art.30311.

Transcriptome-wide gene regulation by gentle treadmill walking during the progression of monoiodoacetate induced arthritis

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Abstract

Objective—Physiotherapies are the most widely recommended conservative treatment options for arthritic diseases. Here we examined the molecular mechanisms underlying the effects of gentle treadmill walking (*GTW*) on various stages of monoiodoacetate-induced arthritis (*MIA*) to unravel the basis for the success or failure of such therapies on the damaged joints.

Methods—Rat knees were harvested from untreated control, MIA afflicted but not subjected to *GTW*, *GTW* regimens started one day post-MIA induction, or after cartilage damage had progressed to Grade 1 or Grade 2. The cartilage was examined by macroscopic, microscopic, μCT imaging and transcriptome-wide gene expression analysis. Microarray data was analyzed by Ingenuity Pathways Analysis to construct molecular functional networks regulated by *GTW*.

Results—*GTW* intervention started on day 1 post-*MIA* induction significantly prevented the *MIA* progression, but its efficacy was reduced when implemented on the knees exhibiting close to Grade 1 cartilage damage. However, *GTW* accelerated damage in the knees with close to Grade 2 cartilage pathologies. Transcriptome-wide gene expression analysis revealed that *GTW* intervention started one day post-*MIA* inception significantly suppressed inflammation-associated genes and upregulated matrix associated gene networks. However, delayed *GTW* intervention following Grade 1 damage was less effective in suppressing proinflammatory genes or upregulating matrix synthesis.

Conclusion—The findings suggest that *GTW* suppresses proinflammatory gene networks and upregulates matrix synthesis to prevent progression of cartilage damage in *MIA* afflicted knees. However, the extent of cartilage damage at the initiation of *GTW* may be an important determinant for the success or failure of such therapies.

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All authors did not have any other financial interests that may create a potential conflict of interest or the appearance of a conflict of interest with regard to the present work.

Exercise is the most widely recommended and used conservative therapeutic approach to improve joint function in arthritis (1–3). Recent guidelines published by Osteoarthritis Research Society International suggest that exercise is in general beneficial for patients with osteoarthritis (OA) (3). Such recommendations are supported by large cohort studies demonstrating that adults engaging in minimal to no physical activity show higher incidence of radiographically diagnosed osteoarthritis (4). Additionally, older adults engaged in moderate physical activity show reduced risk of arthritis-related disability (5). However, in some patients physical activity is either associated with a greater risk or has no effect on knee joints, making it difficult to discern which patients will or will not benefit from physical therapies (6–9).

Chondrocytes, mechanoresponsive cells within the cartilage, perceive and respond to mechanical stimuli by altering their biosynthetic ability, morphology and cartilage extracellular matrix (10,11). These cells interpret mechanical signals in a magnitude dependent manner (12,13). Excessive loading of joints is injurious and activates proinflammatory signaling cascades, similar to those implicated in the etiology of OA (14–17). Contrarily, physiological loading is shown to be antiinflammatory and induce IL-10 production in the synovium, and upregulate synthesis of glycosaminoglycans in the cartilage of patients at increased risk of OA (18–21). In fact, exercise is vital for cartilage homeostasis and its lack atrophies cartilage (22), implicating distinct roles of physiotherapies in both preventing damage and improving joint function.

The aim of this study was to determine the molecular mechanisms underlying the effectiveness of exercise in the form of gentle treadmill walking (*GTW)* on well-defined stages of cartilage damage, using a rat knee model of *MIA* (23). Others and we have shown earlier that low or physiological levels of compressive/tensile forces are antiinflammatory on chondrocytes *in vitro*. These forces suppress several biomarkers of inflammation, such as IL-1β, TNF-α, matrix metalloproteinases (MMPs) and aggrecanases (12,13,24,25). Here, we systematically examined the efficacy of moderate exercise on the progression of *MIA* macroscopically, microscopically, and by microtomographic (μCT) imaging. A transcriptome-wide microarray analysis was also conducted to track the changes in gene expression subsequent to *GTW* therapy to gain key insights into the basis of success or failure of such therapies (1,3,4,26).

MATERIALS AND METHODS

Induction of experimental knee *MIA* and *GTW* regimens

Institutional Animal Care and Use Committee at The Ohio State University approved all protocols. A well-established model of *MIA* in rat knees was used, which exhibited similar pathologies as described by Guzman et al (23). Typically, monoiodoacetate-administered knees exhibited close to Grade 1 cartilage damage on the condylar surface on day 5, close to Grade 2 on condyles by day 9, and close to Grade 3 to 3.5 cartilage and bone damage on day 21 (23).

MIA was induced in the right knees of 12–14 weeks old female Sprague-Dawley rats (Harlan, IN) *via* single intraarticular injection of monoiodoacetate (2 mg/50μl saline/knee) (23). Sham controls (*Cont*) were injected with 50μl saline in the right knees of separate rats. The rats were subjected to *GTW* on a small animal treadmill (Columbus Instruments, OH) at a speed of 12 meters/minute for 45 minutes/day (roughly 0.5 kilometer). This regimen was based on earlier studies, but was gentler to avoid pain and resistance to walking due to *MIA* (27). The *MIA* afflicted rats showed no signs of limping, pain or resistance during *GTW*. One day post-*MIA* induction, the pH of the synovial fluid was between 7.5–8.5, suggesting likely dissemination of monoiodoacetate from joints, prior to start of *GTW* regimens. *GTW*

regimens were started on day 1 (no apparent cartilage damage and less than 2% cell death observed on the surface of cartilage; Live/dead cell kit, Invitrogen, CA), day 5 (close to Grade 1 cartilage damage), and day 9 (close to Grade 2 cartilage damage) post-monoiodoacetate intraarticular injections (Figure 1A, B). On day 21, all rats were euthanized two hours after the last exercise and their knees harvested. The cartilage damage in rats subjected to *GTW* was compared to cartilage damage in non-exercised rats on day 21 post-*MIA* inception. Rats were randomly assigned to 5 groups (n=15 rats/group): *Cont*, saline injected non-exercised sham controls; *MIA21*, *MIA* induced on day 0 and sacrificed on day 21, not exercised; *MIA+GTW1-21*, *MIA* induced on day 0, and subjected to *GTW* daily from days 1 to 21; *MIA+GTW5-21*, *MIA* induced on day 0, and subjected to *GTW* from day 5 to 21; *MIA+GTW9-21*, *MIA* induced on day 0, and subjected to *GTW* from day 9 to 21 (Figure 1A). In each group, femurs from 5 rats were snap-frozen in liquid nitrogen for molecular analysis, and femurs from 10 rats were fixed in 10% buffered formalin for macroscopic, microscopic or μCT imaging analyses. The cartilage damage was graded as described by Pritzker et al (28).

RNA Extraction and Microarray Analysis—The cartilages from the distal end of individual femurs were examined under a stereomicroscope (Zeiss, Germany). Using a scalpel blade, cartilage from the distal end of femurs was carefully sliced off into small chips while maintained in a frozen state, avoiding the areas immediately around lesions to exclude tissue ingrowth in the lesions. The cartilage chips (approximately10 mg/femur) from individual femur were separately collected, and pulverized in a Mikrodismembrator-S (Sartorious, France) at 2500 rpm for 30 seconds. RNA was extracted with Trizol reagent (Invitrogen, CA) (29), and analyzed in a 2100 Bioanalyzer (Agilent, CA) to ensure integrity.

RNA (300 ng) from three independent samples/group was used for cDNA synthesis and labeling using GeneChip Whole Transcript (WT) cDNA Synthesis and Amplification Kit, and GeneChip WT Terminal Labeling Kit, respectively (Affymetrix, CA). The labeled cDNA samples were hybridized on Affymetrix GeneChips Rat Gene 1.0 ST Array and scanned at the Microarray Shared Resource Facility at the OSU.

The intensity scans from three independent GeneChips per treatment group were subjected to gene expression analysis using Partek Genomic Suite version 6.4 (Partek Inc., MO). The significance of differences among the groups was calculated by the analysis of variance (ANOVA) and only significantly differentially regulated transcripts ($p < 0.05$) were considered for further analyses. Variations among the samples in each group were examined by Principal Components Analysis (PCA), and subjected to hierarchical and partition clustering by Partek Genomic Suite.

Functional Gene Network Analysis—The gene expression data derived from microarray analysis was subjected to Ingenuity Pathways Analysis (IPA, Ingenuity Systems, CA) to generate functional molecular networks. A fold-change cutoff of 2.0 was set to identify and assign the molecules to the Ingenuity's Knowledge Base. The gene expression changes were considered in the context of physical, transcriptional or enzymatic interactions of the gene/gene products, and then grouped according to interacting gene networks. A detailed methodology to generate functional molecular relationships involving differentially regulated anabolic and catabolic networks is described in the *Results* section.

Validation of salient genes differentially expressed in the cluster analysis—Expression of selected genes from cluster analysis was confirmed by real-time (rt)-PCR (13). Briefly, first strand cDNA was synthesized from RNA using the Superscript III Reverse Transcriptase Kit (Invitrogen, CA). Gene expression was assessed by amplifying the cDNA with custom-designed primers using the iCycler iQ Real-Time PCR System (Bio-

Rad, CA). The primers used were: *Rps18-sense* 5′-GCGGCGGAAAATAGCCTTCG-3′; *Rps18-anti-sense* 5′-GGCCAGTGGTCTTGGTGTGCTG-3′; *Fcgr1a-sense* 5′-AGCGGCATCTATCACTGCTCA-3′; *Fcgr1a-anti-sense* 5′-TCAGCACTGGTGTGGCAAATA-3′; *Aspn-sense* 5′-CAAAGAGCCAGTGAACCCCTT-3′; *Aspn-anti-sense* 5′-TCAGAACAGTGGACGACTCGA-3′; *Mmp12-sense* 5′-CCAGGAAATGCAGCAGTTCTTT-3′; *Mmp12-anti-sense* 5′-GCTGTACATCAGGCACTCCACAT-3′; *Alox5-sense* 5′-TTCTCCGCACACATCTGGTGT-3′; *Alox5-anti-sense* 5′-GGCAATGGTGAACCTCACATG-3′; *Vcam1-sense* 5′-GCCGGTCATGGTCAAGTGTTT-3′; *Vcam1-anti-sense* 5′-CATGAGACGGTCACCCTTGAA-3′; *Cilp-sense* 5′-TGTGAAGTCCAAGGTCACCCA-3′; *Cilp-anti-sense* 5′-GTAGAAGGAGTTGGTGGCATTCTG-3′; *Sox9-sense* 5′-ATCTGAAGAAGGAGAGCGAG-3′; *Sox9-anti-sense* 5′-CAAGCTCTGGAGACTGCTGA-3′; *Col9a1-sense* 5′-TGATGGCTTTGCTGTGCTG-3′; *Col9a1-anti-sense* 5′-TGACTGGCAGTTCATGGCA-3′; *Frzb- sense* 5′-TGCCCTCCCCTCAGTGTTAAT-3′; *Frzb-anti-sense* 5′-CAAGCCGATCCTTCCACTTCT-3′; *Col2a1-sense* 5′-ATGAGGGCCGAGGGCAACAG-3′; *Col2a1-anti-sense* 5′-GATGTCCATGGGTGCAATGTCAA-3′.

Statistical Analysis

The significance among the conditions in the microarray data was tested by Partek Genomic suite by ANOVA among the experimental groups (n=3). ANOVA with Tukey's HSD post-hoc test by SPSS v17 was used to determine the significance levels of rt-PCR data that include two additional independent samples per group to microarray-examined specimens (n = 5). $p < 0.05$ was regarded as significant.

RESULTS

GTW prevents progression of cartilage destruction when implemented at the early stages of *MIA*

Comparing the anatomy/morphology of *MIA+GTW1-21* to non-exercised *MIA21* femoral cartilage revealed that exercise significantly prevented progression of MIA. This was evident from smooth surface, minimal aberrations or lesions on the cartilage surface of *MIA +GTW1-21*. The histological examination of *MIA+GTW1-21* cartilage revealed slight thinning of cartilage in small areas, whereas cartilage and subchondral bone was preserved with no signs of bony changes (Figure 1Ce–h). The μCT images confirmed that early intervention by exercise prevented bone erosion as compared to non-exercised cartilage of *MIA21* (Figure 1Ct).

We next determined whether exercise could prevent or delay progression of MIA in rats with close to Grade 1 cartilage damage. Analysis of *MIA+GTW5-21* femurs revealed that *GTW* appeared to delay the progression of MIA, as evident by the relatively smooth condylar surface and absence of abrasions (Figure 1Ci–l). Histological analysis showed close to Grade 1.5–2 damage, as compared to non-exercised *MIA21* cartilage showing Grade 3–3.5 damage. In parallel, μCT images showed reduced severity of bone erosion in *MIA +GTW5-21* femurs (Figure 1Cl). Contrarily, initiation of *GTW* 9 days following inception of MIA (*MIA+GTW9-21*), resulted in close to Grade 4 or greater damage, showing denuded cartilage and sclerotic subchondral bone that covered the femoral condyles, patellar groove

and ridges. Imaging by μCT also confirmed greater bone loss on both the femoral condyles and patellar groves (Figure 1Cp), when compared to bone damage in *MIA21* (Figure 1Ct).

Extent of cartilage damage at the inception of *GTW* critically influences the expression of catabolic and anabolic genes

The RNA from the femoral cartilage of rats in *MIA21*, *MIA+GTW1-21* and *MIA+GTW5-21* were subjected to microarray analysis and their gene expression compared. Due to limited cartilage remaining on the condyles, *MIA+GTW9-21* samples were excluded from this analysis. PCA revealed significantly distinct distribution of gene expression among samples in each group (n=3) (Figure 2A), as evident by the average F ratio (signal to noise ratio) of 15.5 in a total of 27,342 transcripts on the Affymetrix GeneChips array. The hierarchical clustering of the differentially regulated genes (2 fold change, $p<0.05$) showed that: (i) *Cont* cartilage showed minimal active genes (red) and maximal number of quiescent genes (blue); (ii) *MIA21* regulated maximal number (1179, 4.29%) of transcripts; (iii) *MIA+GTW1-21* regulated 847 (3.09%) transcripts, with gene expression pattern closer to that of *Cont*; (iv) *MIA+GTW5-21* regulated 1103 (4.03%) transcripts, with gene expression pattern closer to *MIA21* (Figure 2B).

MIA afflicted knees showed a temporal gene regulation pattern during the progression of cartilage damage (Figure 2C). The differentially expressed genes identified here could be categorized into 5 clusters: *Cluster I*, immune responses and innate immunity showing peak-upregulation in cartilage with Grade 1 damage (day 5 after MIA inception); *Cluster II*, chronic immune responses and immune trafficking showing peak-upregulation in cartilage with close to Grade 2 damage (day 9); *Cluster III*, chronic inflammatory diseases and immune suppression/adaptation exhibiting gradual increase in gene expression until cartilage showed close to Grade 3 to 3.5 damage (day 21); *Cluster IV*, musculoskeletal development and function associated genes showing peak downregulation in cartilage with Grade 1 damage (day 5); and *Cluster V*, genetic disorders and skeletal and musculoskeletal diseases associated genes showing peak downregulation in cartilage with Grade 2 damage (day 9). We next examined the effects of *GTW* as a mode of exercise on genes in each *Cluster* with respect to those in *MIA21* (Figure 2D). Intervention by *GTW* in *MIA+GTW1-21* suppressed approximately 52%, 50% and 59% of the genes upregulated in *MIA21* in the inflammatory *Clusters I, II and III*, respectively. In parallel, *GTW* upregulated approximately 33% and 31% of the genes that were suppressed in *MIA21*, in *Clusters IV and V*, respectively. However, when exercise was initiated after Grade 1 cartilage damage *(MIA +GTW5-21)*, the suppression of genes in *Clusters I, II*, and *III* was only 6%, 14% and 32%, respectively. Similarly, less than 3% genes in *Cluster IV* and 11% genes in *Cluster V* were upregulated in *MIA+GTW5-21* (Figure 2D).

Table 1 shows salient differentially regulated genes by various exercise regimens (*MIA +GTW1-21 and MIA+GTW5-21*) as compared to *MIA21*. The major genes suppressed by *GTW* in *MIA+GTW1-21* in *Cluster I* were *Alox5ap* (arachidonate 5-lipooxygenase activating protein required for Alox activation), *Fcgr1a* (*Cd64*, high affinity immunoglobulin gamma Fc receptor I, regulates innate/specific immune responses), *Hla-dmb* (HLA class II antigen beta-chain), *Cd53* (surface molecule regulates innate levels of TNF-α), *Aspn* (asporin, negatively regulates TGF-β), *Calcr* (calcitonin receptor, involved in bone formation), *Ctsg* (cathepsin G, a peptidase), and *Il1rl1* (IL-1 receptor like-1). The *Cluster II* genes downregulated by *GTW* were *Cd84* (adherence associated molecule), *Il-18* (Interleukin-18), *Mmp-12* (elastase), *Mmp-19* (involved in tissue remodeling), *Adamts4* (aggrecanase), *Adamts7* (degrades cartilage oligomeric protein), *Ccr1* (chemokine receptor 1, chemoattracts cells), and *Ccl9* (osteoclast activation through *Ccr1*). The genes suppressed in *Cluster III* included *Alox5*, *Clec4d* (C-type lectin domain family 4 involved in antigen uptake), *Vcam1* (vascular cell adhesion molecule-1), *Adam23* (disintegrin and metalloproteinase domain-

containing protein 23 involved in cell adhesion), *Postn* (periostin, involved in bone formation), and *Crlf1* (cytokine receptor-like factor 1), all of which are involved in chronic inflammation. More importantly, in *Clusters IV* and *V*, *GTW* upregulated expression of extracellular matrix associated genes that were suppressed in *MIA21*. *Cluster IV* included *Cilp* (cartilage intermediate layer protein), *Cilp2*, *Acan* (aggrecan), *Sox9* (transcription factor required for chondrocyte matrix proteins), *Cytl1* (cytokine like-1, promotes proteoglycan synthesis), *Crlf1* (cytokine receptor-like factor 1), *Igf2* (insulin like growth factor II, chondrocyte growth and differentiation). Similarly, genes upregulated by *GTW* in *Cluster V* were Collagens (*Col2a1*, *Col9a1*, *Col9a2*, *Col9a3*, *Col11a2*), *Matn3* (matrilin 3), *Frzb* (Wnt signaling inhibitor), *Mia* (melanoma-derived growth regulatory protein), *Chad* (chondroadherin, mediates chondrocyte adhesion), *Hapln1* (hyaluronan and proteoglycan link protein 1), *Vit* (vitrin, promotes matrix assembly) and *Prg4* (Lubricin). On the other hand, cartilage from *MIA+GTW5-21* exhibited that *GTW* only partially upregulated genes suppressed in *MIA21*.

The extent of cartilage damage at the initiation of GTW determines its effectiveness through the differential regulation of major intracellular pathways

Among 1179 genes regulated more than 2-fold ($p<0.05$) in *MIA21*, 142 genes were included in human and experimental 'arthritis' disease category in the Ingenuity's Knowledge Database. These 142 genes clustered to show the general trends regulated by exercise. This analysis showed that intervention by *GTW* in *MIA+GTW1-21* suppressed 96% genes that are upregulated in *MIA21* (red in the intensity plot), and upregulated 81% genes that are downregulated in *MIA21* (green in the intensity plot), as evident by color shifting toward darker shades, i.e., closer to control levels. Contrarily, *GTW* when initiated after 5 days of the onset of MIA (*MIA+GTW5-21)*, suppressed 80% genes upregulated in *MIA21*, and upregulated only 58% genes that are downregulated in *MIA21* (Figure 3A).

During the progression of MIA, NF-*κ*B and TGF-*β* play a focal role in regulating gene expression in the inflammatory *Clusters I, II* and *III* and the anabolic *Clusters IV* and *V*. Consequently, the functional relationships among NF-*κ*B, TGF-*β* and 142 arthritis-related genes were analyzed using an IPA custom molecular network generation tool (Network Explorer) (Figure 3B–E). This analysis revealed that the catabolic gene networks induced during the progression of MIA, were downregulated (green symbols) by *GTW* in *MIA +GTW1-21* likely *via* suppression of NF-κB activity, a major node in this network (Figure 3B). The NF-κB in turn may regulate arachidonate-lipoxygenase pathway (*Alox5, Alox5ap*), adherence molecules (*Itgb1, Itgal, Vcam1, Cd44*), cell cycle associated genes (*Ck6, Cdkn1a, Bcl2, Ank1*), cytokines *(Il-18, Il-15)*, and MMPs (*Mmp12, Mmp14*). Similarly *GTW* in *MIA +GTW1-21* upregulated anabolic gene networks *via Tgf-β* and *Sox9*. These growth factors and transcription factors have been shown to regulate the expression of Collagens (Col type IIα1, Col type IXα1, Col type IX α2, Col type IX α3, Col type XI α2), *Cilp* (cartilage intermediate layer protein), *Cilp2*, *Mgp* (matrix gla protein), *Acan* (Aggrecan), and other matrix proteins (Figure 3C red symbols; Table 1). The same arthritis-associated networks in *MIA+GTW5-21* demonstrated that intervention by *GTW* even after Grade 1 cartilage damage, suppressed several genes associated with NF-κB activity (Figure 3D). Contrarily, several proinflammatory genes such as *Aspn*, *Mmp-12*, *Ccl9*, interferon regulatory factor 5 (*Irf5*), integrin beta 2 (*Itgb2*), and cathepsin G *(Ctsg*), periostin (*Postn*) were not suppressed or upregulated by *GTW* when implemented following Grade 1 cartilage damage (Figure 3E). Additionally, in *MIA+GTW5-21, Sox9* was further suppressed and together with Tgf-β likely led to the downregulation of *Acan, Alp, Cilp, Cilp2*, and *Mgp* required for matrix assembly. Nevertheless, the expression of Collagens (*ColIXa1, ColIXa2, ColIXa3, ColIIa1, ColXIa2*) was upregulated in *MIA+GTW5-21 as* compared to *MIA21* (Figure 3E).

Real-time-PCR validated the microarray findings showing differential downregulation of the salient genes in *Clusters I, II* and *III* (*Fcgr1a, Aspn, Mmp12, Alox5, Vcam1*), and upregulation of the genes in *Clusters IV* and *V* (*Cilp, Sox9, Col9a1, Frzb, Col2a1*) by *GTW* in *MIA+GTW1-21* and *MIA+GTW5-21* (Figure 4A). The IPA showed that the regulation of NF-κB may play a focal role in the antiinflammatory effects of *GTW*. Since NF-κB activity in the cells is shown to be oscillatory (30, 31) and thus may not provide the true activation state in the inflamed knees, we examined the expression of several signaling molecules in the NF-κB pathway. As shown in Figure 4B, *GTW* in *MIA+GTW1-21* suppressed *Traf2* (TNF receptor associated factor 2), *Traf3, Traf6, Tank* (TRAF family member-associated NF-kappa-B activator), *Ripk1* (Receptor (TNFRSF) interacting Ser-Thr kinase), *Ripk3,* and *Ikbkg* (I-κb kinase γ/IKKγ) expression, that were upregulated in *MIA* afflicted knees. However, GTW prevented expression of fewer MIA-induced genes, e.g., *Traf3, Tank, Ripk3,* and *Ikbkg* when it was initiated in rats with Grade 1 cartilage damage (Figure 4B).

DISCUSSION

The present study documents the effects of *GTW* as a form of exercise on the global gene regulation in the articular cartilage of the knee afflicted with various stages of *MIA*. In *MIA*, acute and chronic inflammations drive the destruction of the knee, whereas inhibition of matrix synthesis and its breakdown prevent the repair that worsens the joint damage (32–35). We showed that *GTW* suppresses inflammation and upregulates repair to prevent active progression of cartilage damage. Nevertheless, the maximal effects were observed when *GTW* was implemented on the knees with Grade 1 or lesser cartilage damage. Contrarily, when the knees with Grade 2 damage were subjected to *GTW*, its effectiveness was compromised, and the cartilage damage was further intensified as compared to *MIA21*. Benefits of exercise in the form of *GTW* are likely contingent upon many factors including adherence to exercise regimens, frequency of exercise, speed of walking, range of motion, and actual loading of the symptomatic compartment. Furthermore, efficacy of exercise on various stages of arthritic lesions is less predictable, mainly due to the limitations in detecting the extent of cartilage damage in humans (36–38). In the present model of MIA, our observations indicate that the extent of cartilage damage may also play an important role in achieving the optimal effects of gentle exercise.

The major limitation of the MIA model used in the present study is that monoiodoacetate induces aggressive cartilage destruction, which progresses to Grade 3 to 3.5 damage within 21 days. Thus, these lesions may not depict cartilage damage caused by trauma/insult that takes extended periods of time in human OA (23). This is an important limitation that must be considered when trying to extend/translate these findings to humans with arthritis. Nevertheless, it is important to note that even during this aggressive progression of cartilage destruction, *GTW* could suppress progression of inflammation and cartilage loss, as evident from macroscopic, microscopic and μCT imaging. In support of exercise-mediated joint function, it is recently reported in a cohort of 2589 OA patients that the extent of physical activity and better joint performance are proportionally related (39).

Presently, both resistance and gentle exercises are prescribed for rehabilitation of injured or arthritic knees. However, the optimal duration and physical loading necessary for achieving the beneficial effects of exercise are unclear. In the present study, we subjected knees to 45 minutes of *GTW*, at a rate of 12 meters/minute. Considering the aggressive nature of the MIA model used, the selected speed and duration of *GTW* were gentler than those used in the earlier experimental models, *e.g.*, 16 meters/min for 1h (27). The effects of longer or shorter duration or different speeds of *GTW* are yet to be determined. For example, a different exercise regimen on *MIA+GTW5-21* may have potentially prevented cartilage damage more effectively. Interestingly, even a single bout of exercise (90° knee bending for

250 times) is shown to increase IL-10 and suppress release of cartilage oligomeric protein and aggrecan levels in intra-articular and peri-synovial fluids from osteoarthritic knees (20). These findings further support that exercise may act as an antiinflammatory and reparative signal on inflamed knees.

The information gained from the transcriptome-wide gene expression analysis demonstrated that counteracting the MIA induced gene induction or suppression appears to be a primary mechanism underlying the *GTW*-mediated inhibition of cartilage damage. *GTW* suppresses expression of significant number of innate and chronic immunity related genes (*Clusters I, II and III)* demonstrating the potential of such exercises in suppressing inflammation. Additionally, *GTW* promotes repair by inducing the expression of genes related to musculoskeletal development and functions (*Clusters IV* and *V)* in *MIA+GTW1-21*. It is likely that, the partial success of *GTW* in limiting the existing cartilage damage in *MIA +GTW5-21* may be related to its inability to inhibit/induce expression of some of these genes in catabolic and anabolic clusters. Specifically, NF- κB controls proinflammatory gene induction and plays a critical role during cartilage inflammation (24,40–42). According to IPA network explorer, intervention by exercise in *MIA+GTW1-21* may suppress NF-κB activity and thus genes associated with the NF-κB networks, such as those involved in apoptosis and cell cycle *(Cdkn1a and Bcl2)*, cell adhesion *(Itgb* and *Vcam)*, complement *C3*, matrix breakdown *(Mmp-12 and Mmp-14)*, and proinflammatory responses (*Il-15* and *Il-18)* (43–45). Interestingly, GTW suppressed fewer genes in the NF-kB signaling cascade that was also reflected in the lesser extent and number of proinflammatory genes suppressed in MIA+GTW5-21.

During inflammation, activation of proinflammatory cytokine receptors leads to sequential activation of receptor associated kinases, adaptor proteins, TRAFs, and IKK complex (IKKα and IKKβ kinases and IKKγ) which ultimately determines NF-κB activity (13). Figure 5 shows differential regulation of signaling molecules and gene expression when *GTW* was implemented 1 day or 5 days after the onset of MIA. For example, *Ikbkg (IKKγ), Traf2, Traf3, Traf6, Tank* and *Ripk* essential to activate IKK complex were significantly suppressed by *GTW* initiated on day 1 post-MIA induction. Expression of *Irak4*, which is required to recruit TRAF6 into the signaling complex, was also suppressed by exercise in *MIA +GTW1-21*. Additionally, RIP kinases that activate RIP to bind IKKγ and recruit it to the TNFR1 signaling complex independent of TRAF, are also suppressed in *MIA+GTW1-21* (30). These findings further suggest that exercise may collectively suppress gene expression required for NF-κB activity and thus inflammation. In this context, the inability of *GTW* in *MIA+GTW5-21* to suppress *Traf3, Tank, Ripk1, Ripk3*, and *Ikbkg* may be responsible for only partial prevention of the progression of *MIA* (Figure 5). Previous *in-vitro* studies showing that antiinflammatory actions of mechanical signals are mediated by suppression of NF-κB activation via TAK1, IKK, and I-κB also support the present findings (13,24,40,42). Additionally, the antiinflammatory effects of exercise have been shown in arthritis and other conditions. For example, exercise is shown to increase levels of antiinflammatory cytokine IL-10 in the intra-articular and peri-synovial fluids of patients with osteoarthritis (19), suppress low-grade systemic inflammation (20), decrease inflammation in diabetes (46), and suppress IL-8, C reactive protein and interferon-γ in fibromyalgia patients (47). These findings demonstrate that in addition to local effects, exercise exerts systemic effects. Whether exercise also suppresses systemic markers associated with cartilage damage in *MIA* is yet to be determined.

In the context of *GTW* preventing matrix loss, the schematic in Figure 5 shows the regulation of signaling molecules and matrix proteins by *GTW* in MIA afflicted cartilage. *MIA* significantly upregulates *Aspn*, a known inhibitor of TGF-β (48). Strikingly, *GTW* significantly suppresses *Aspn* expression with parallel upregulation of molecules in TGF-β

complex in *MIA+GTW1-21* cartilage. The anabolic networks of TGF-β may upregulate *Sox9* and together may serve as focal points for the significant upregulation of many chondrocytic matrix associated genes such as aggrecan, collagens, *Cilp, Cilp2, Matn3* and *Vit* by exercise in *MIA+GTW1-21*. Contrarily, failure of asporin suppression in *MIA+GTW5-21* may be responsible for the partial repair of MIA-afflicted cartilage. This dynamic downregulation of TGF-β via *Aspn* induction in *MIA21* and counter regulation by *GTW* suggests an important role of *Aspn* in exercise-mediated anabolic responses in cartilage (48,49). Interestingly, cartilage is believed to have very limited capacity to regenerate/repair. The present findings are novel in showing that exercise such as *GTW* can augment anabolic gene expression to prevent cartilage loss and matrix restructuring in inflamed cartilage.

Overall, the present study is the first to delineate the molecular basis for the efficacy of *GTW* in suppressing progression of cartilage destruction. We show that *GTW* is a robust regulator of anti-catabolic and anabolic networks that suppress inflammation and upregulate matrix synthesis, even during actively progressing cartilage destruction. Importantly, the effects of exercise appear to be inversely related to the extent of cartilage damage, i.e., its implementation at the earlier stages of cartilage damage may provide the greater benefits. Further studies are required to understand how these robust therapeutic effects of exercise can be optimized to prevent cartilage destruction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grant number AR048781 from the National Institute of Arthritis, Musculoskeletal and Skin Diseases, DE015399 and DE014320 from the National Institute of Craniofacial and Dental Research at the National Institute of Health, Bethesda, MD.

Financial Support information. Authors acknowledge that no financial support or other benefits from commercial sources was received for the work reported in the manuscript.

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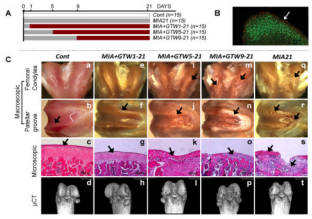

Figure 1.

Effects of *GTW* on the progression of MIA. (A) Experimental scheme. (B) Cartilage section showing dead (red) and live (green) cells on day 1 post-MIA inception. (C) Macroscopic, microscopic and bone imaging by μCT of: healthy sham control femur showing smooth surface, normal histology and no bone lesions on the femoral condyles and patellar grove (*see* Supplemental Data for 360° μCT projections) (a – d); *MIA+GTW1-21* cartilage showing no surface abrasions on the condyles, some cartilage lesions on the patellar groove and ridges, near normal histology, and no bone involvement by μCT images (e – h); *MIA +GTW5-21* cartilage showing some abrasions on condyles, cartilage damage on patellar groove and ridges, H&E section showing focal matrix condensation, cell clustering and disorganization, fibrocartilage formation, and some bone lesions by μCT images (i – l); *MIA +GTW9-21* cartilage demonstrating extensive cartilage lesions on condyles and patellar groove, severe cartilage loss, denuded bone, and excessive bone lesions on femoral condyles and patellar grove in μCT images (m – p); *MIA21* cartilage exhibiting cartilage matrix loss, delamination of superior surface, excavation and matrix loss in superficial and mid zone (q – t). Each femur is representative of each group showing similar characteristics (n=10).

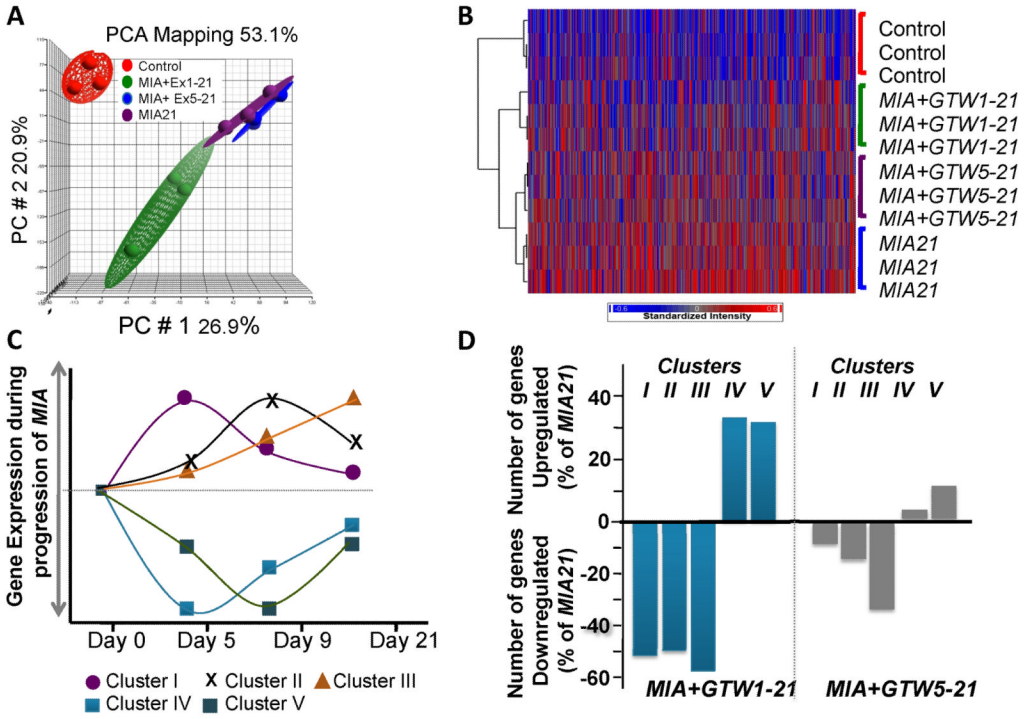

Figure 2.

Transcriptome-wide microarray analysis of femoral cartilage from *Cont* healthy joints or from *MIA+GTW1-21*, *MIA+GTW5-21* or *MIA21* joints. (A) PCA analysis showing reproducible gene expression in the articular cartilage from the right knee joint of 3 separate rats from *Cont*, *MIA+Ex1-21*, *MIA+GTW5-21* or *MIA21*. (B) Overall gene expression profiles of the articular cartilage of 3 separate rats from *Cont*, *MIA+GTW1-21*, *MIA +GTW5-21* or *MIA21* groups. Intensity plot with dendrogram represents the transcripts that were significantly ($p < 0.05$) and differentially upregulated (red) or downregulated (blue) by more than two-fold. The analysis shows the most differential changes in gene expression in *MIA+GTW1-21*, followed by *MIA+GTW5-21* as compared to gene expression in *MIA21* cartilage. (C) Temporal regulation of *MIA* associated Gene Clusters associated with: acute and innate immune responses (*Cluster I*); chronic inflammatory responses and genetic disorders (*Cluster II*); musculoskeletal disorders and inflammatory diseases (*Cluster III*); and genetic disorders and skeletal and muscular disorders (*Cluster IV and V*). (D) The percentage of genes that were significantly ($p<0.05$) up or downregulated by exercise (*MIA +GTW1-21* and *MIA+GTW5-21)* in gene *Clusters I, II, III, IV* and *V* in comparison to genes regulated in *MIA21*.

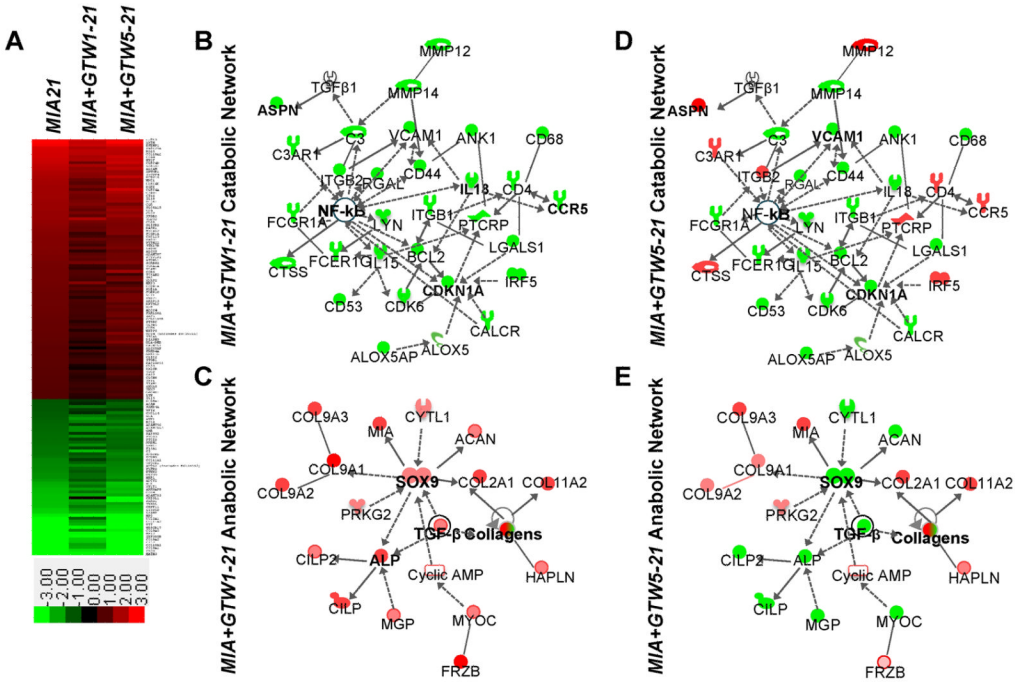

Figure 3.

Catabolic and anabolic networks regulated by *GTW* in *MIA*. (A) Intensity plot of 142 genes that are a known set of arthritis-associated genes in Ingenuity Knowledge Database, and more than 2-fold up (red) or down (green) regulated in *MIA21* (*see* Supplemental Data for details). (B–E) IPA generated networks of: (B) catabolic genes that are downregulated by *GTW* in *MIA+GTW1-21* showing suppression of genes involved in acute and chronic inflammatory/immune responses via the suppression of NF-κB activity; (C) anabolic genes that are upregulated by exercise in *MIA+GTW1-21* showing induction of *Sox9* and TGF-β that in turn may upregulate the expression of matrix associated genes; (D) catabolic gene regulation by *GTW* in *MIA+GTW5-21* showing partial suppression of genes involved in acute and chronic immune responses; (E) anabolic gene regulation by *GTW* in *MIA +GTW5-21* showing suppression of *Sox9* and TGF-β and number of matrix proteins that may limit its ability to repair cartilage. Red, green, and white colors represent upregulation, downregulation and no regulation, respectively. The shading of colors represents dark, greater changes to light lesser changes. Symbols are: cytokine/growth factor, phosphatase, Transcription regulator, translation regulator, transmembrane receptor, complex group, enzyme, G protein coupled receptor, kinase, peptidase, and other.

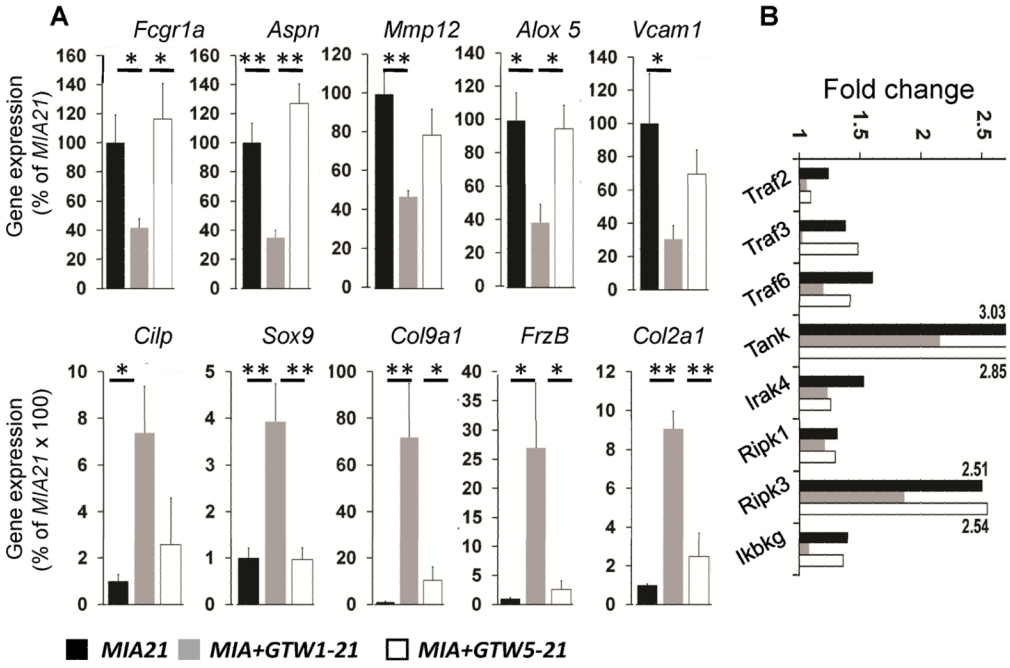

Figure 4.

(A) Genes selected from each *Cluster* to demonstrate their up- or downregulation by quantitative rt-PCR analysis in *MIA+GTW1-21* or *MIA+GTW5-21* cartilage, as compared to *MIA21* cartilage. *Fcgr1a* (IgG Fc receptor gamma 1a), *Aspn* (Asporin), *Mmp12* (Matrix metalloproteinase 12), *Alox5* (Arachidonate-5-lipoxygenase), *Vcam1* (vascular endothelial cell adhesion molecule 1), *Cilp* (cartilage intermediate layer protein), *Sox9* (Sry-related HMG box -9), *Col9α1* (Collagen type 9α1), *Frzb* (Frizzled B), *Col2α1* (Collagen type 2α1). Data represents rt-PCR analysis performed on RNA from five separate rats in each group. The * and ** indicate significance values of $p > 0.05$ and $p > 0.01$, respectively. (B) Regulation of the genes required for the activation of NF-κB by *GTW*. Suppression of *Traf2* (TNF receptor associated factor), *Traf3, Traf6, Tank* (TRAF family member-associated NF-kappa-B activator), *Ripk1* (Receptor (TNFRSF) interacting Ser-Thr kinase), *Ripk3*, *Ikbkg* (I-κb kinase γ/IKK γ) expression in *MIA+GTW1-21and MIA+GTW5-21*, as compared to *MIA21* in the microarray data. Upregulation of the gene expression in *MIA21* was compared to *Cont*. Each point represents mean of microarray data derived from 3 independent cartilage specimens ($p<0.05$).

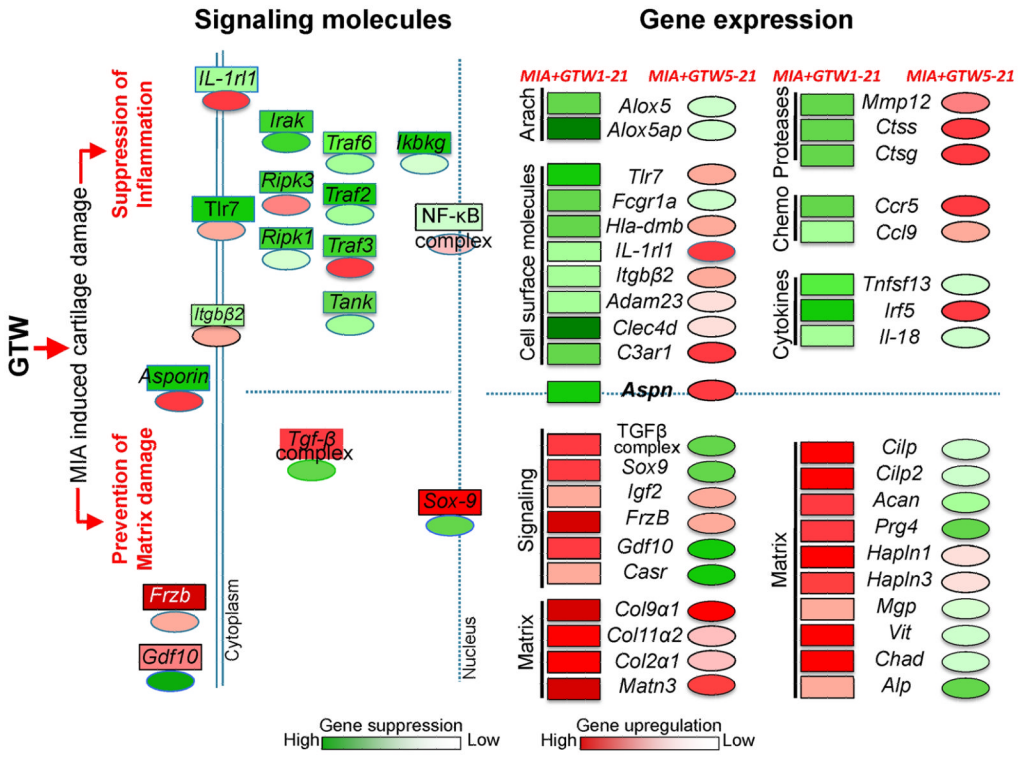

Figure 5.

Schematic showing the molecular basis of the protective effects of *GTW* on *MIA*-induced cartilage damage. *GTW* preserves cartilage integrity by preventing *MIA*-induced inflammation and matrix loss in *MIA+GTW1-21* (rectangles) to a greater extent than in *MIA +GTW5-21* (ovals), where *GTW* was applied following Grade 1 cartilage damage. These differential effects stem from the ability of *GTW* to suppress (green) MIA-induced NF-κB signaling molecules in *MIA+GTW1-21*, as compared to minimal suppression or upregulation (red) of these molecules in *MIA+GTW5-21*. This in turn may suppress several proinflammatory genes [arachidonate metabolites (Arach), receptors, proteases, chemokines (Chemo) and cytokines] in *MIA+GTW1-21*, but to a lesser degree in *MIA+GTW5-21*. Importantly, *Aspn*, an inhibitor of TGF-β that is upregulated in *MIA21*, is significantly suppressed by *GTW* in *MIA+GTW1-21*. By suppressing Asporin expression, *GTW* may upregulate TGF-β complex and SOX-9 in *MIA+GTW1-21*, required for synthesis of matrix proteins. Simultaneous upregulation of Frizzled-related protein (*Frzb*) likely inhibit chondrocyte hypertrophy and mineralization (50). Contrarily, the lack of Asporin suppression and thus failure of the expression of the molecules in TGF-β complex and *Sox-9* in *MIA+GTW5-21* may prevent MIA-induced matrix loss to a lesser extent, as evidenced by the suppression of several molecules required for matrix assembly.

Table 1

GTW-induced differential expression of salient genes in *MIA* afflicted cartilage.

Cluster I		
Gene	***MIA+GTW 1-21 (%MIA21)***	***MIA+GTW 5-21 (%MIA21)***
Alox5ap	−206	−119
Calcr	−183	−136
Fcgr1a	−182	−101
Tlr7	−179	+115
C3	−178	−104
Hla-dmb	−176	+130
Cd53	−169	−122
Aspn	−166	+148
Ctsg	−126	+106
Il1rl1	−124	+156

Cluster II		
Gene	***MIA+GTW1-21 (%MIA21)***	***MIA+GTW 5-21 (%MIA21)***
Cd84	−161	−133
Il18	−149	−102
Mmp12	−144	+130
Cd44	−140	−104
Tnfsf13	−140	−103
Adamts7	−136	−112
Adamts4	−118	−185
Ccl9	−117	+125
Ccr1	−112	−117
Mmp19	−110	−121

Cluster III		
Gene	***MIA+ GTW1-21 (%MIA21)***	***MIA+GTW5-21 (%MIA21)***
Clec4d	−209	+103
Alox5	−193	−126
Vcam1	−171	−102
Adam23	−138	+103
Crlf1	−131	−126
Cdh13	−125	−139
Postn	−122	+123
C1s	−120	−142
Serpine1	−119	+101
Cd14	−115	−152

Cluster IV		
Gene	***MIA+ GTW1-21 (%MIA21)***	***MIA+GTW5-21 (%MIA21)***
Cilp	+445	−104
Cytl1	+226	−107
Cilp2	+210	−156
Hapln3	+158	+103
Acan	+152	−110
Sox9	+150	−123
Gdf10	+140	−176
Igf2	+139	+136
Casr	+139	−219
Chst3	+114	−128

Cluster V		
Gene	***MIA+GTW1-21 (%MIA21)***	***MIA+GTW5-21 (%MIA21)***
Col9a1	+1102	+341
Matn3	+979	+322
Frzb	+761	+129
Mia	+381	+229
Col2a1	+324	+109
Chad	+287	−101
Hapln1	+277	+108
Col11a2	+264	+126
Vit	+251	−101
Prg4	+165	−140