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Synovial expression of Th17-related and cancer-associated genes is regulated by the arthritis severity locus Cia10

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Abstract

We have previously identified Cia10 as an arthritis severity and articular damage quantitative trait locus. In this study we used Illumina RatRef-12 microarrays to analyze the expression of 21,922 genes in synovial tissues from arthritis-susceptible DA and arthritis-protected DA.ACI(Cia10) congenics with pristane-induced arthritis. 310 genes had significantly different expression. The genes up-regulated in DA, and reciprocally down-regulated in DA.ACI(Cia10) included IL-11, Ccl12 and Cxcl10, as well as genes implicated in Th17 responses such as IL-17A, IL-6, Ccr6, Cxcr3 and Stat4. Suppressors of immune responses Tgfb and Vdr, and inhibitors of oxidative stress were up-regulated in congenics. There was an over-representation of genes implicated in cancer and cancer-related phenotypes such as tumor growth and invasion among the differentially expressed genes. Cancer-favoring genes like Ctsd, Ikbke, and Kras were expressed in increased levels in DA, while inhibitors of cancer phenotypes such as Timp2, Reck and Tgfbr3 were increased in DA.ACI(Cia10). These results suggest that Cia10 may control arthritis severity, synovial hyperplasia and joint damage via the regulation of the expression of cancer-related genes, inflammatory mediators and Th17-related markers. These new findings have the potential to generate new targets for therapies aimed at reducing arthritis severity and joint damage in rheumatoid arthritis.

Keywords

rheumatoid; autoimmunity; synovial; erosions; animal model; genetic

INTRODUCTION

Rheumatoid arthritis (RA) is a complex autoimmune disease characterized by chronic joint inflammation that commonly leads to joint destructive changes, disability and reduced

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CONFLICT OF INTEREST

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quality of living. RA affects 1% of the population and is regulated by both genetic and environmental factors [1]. Most of the genetic studies have focused in the identification of susceptibility genes. The HLA-DRB1 shared-epitope association with RA susceptibility has been known for over two decades, and in recent years several non-MHC susceptibility alleles have been identified, including PTPN22 [2], CTLA4 [3], TNFAIP3 and TRAF1 [4, 5]. Yet, little is known about the genetic regulation of disease severity and joint damage, which are among the best predictors of disease outcome and disability.

We have identified the non-MHC arthritis severity and joint damage quantitative trait locus (QTL) Cia10 on rat chromosome 2, studying an intercross between MHC-identical DA (severe arthritis) and ACI (arthritis-resistant) rats [6]. DA.ACI(Cia10) congenic rats, which are identical to DA except for the presence of ACI alleles at the Cia10 interval, were generated using a genotype-guided strategy and found to develop a significantly milder form of pristane-induced arthritis (PIA), and to preserve normal joint architecture compared to DA rats. DA.ACI(Cia10) congenics also had significantly reduced synovial tissue levels of mRNA of pro-inflammatory cytokines central to RA pathogenesis [7]. During the refining of the congenic interval towards positional cloning of the Cia10 gene we aimed to identify molecular pathways and potential candidate genes involved in protecting DA.ACI(Cia10) congenics, using genome-wide microarray gene expression profiling of synovial tissues. In the present study we report the presence of ACI alleles at the Cia10 interval correlates with reduced expression of genes implicated in Th17 responses, including IL-17A. Additionally, congenics had reduced expression of genes involved in the regulation of cancer growth and invasion, which may suggest new targets for therapies aimed at reducing synovial pannus growth and cartilage and bone invasion and destruction.

RESULTS

DA.ACI (Cia10) congenics are protected and developed significantly milder PIA compared with DA

DA.ACI(Cia10) congenic rats developed significantly milder arthritis compared with DA, with a median ASI of 12.3 compared with 30.4, respectively (P=0.002 Mann-Whitney test) (Figure 1B). These observations were in agreement with our previous studies [7].

Gene expression analysis of synovial tissues differentiates DA from DA.ACI(Cia10) congenics

7,593 (34.6%) of the 21,922 genes present in the array were expressed by all 18 synovium samples and were used for analysis. 310 of the 7,593 genes (4.08%) expressed by all synovial tissues met the criteria for differential expression. 120 of these 310 genes (38.7%) were expressed in significantly higher levels in DA.ACI(Cia10) compared with DA rats. 190 of the 310 genes (61.3%) were expressed in significantly increased levels in DA synovium, and were reciprocally reduced in DA.ACI(Cia10).

The genes with the most significantly increased expression in the DA.ACI(Cia10) congenics and reduced expression in DA included Mup4 (4.88-fold, P=0.0067), Aldh1a1 (3.7-fold

P=0.009), Rpesp (3.56-fold, P=0.0082), Igfbp6, (3.56-fold, P=0.0068), Gstm1 (2.58-fold, P=0.0029), Timp2 (2.15-fold, P=0.0024) and Reck (2.1-fold, P=0.005) (table 2).

Among the 190 genes with significantly reduced expression levels in the synovial tissues of DA.ACI(Cia10) congenics and increased expression in DA was Gp49b (5.39-fold, P=0.0023), Cd53 (3.28-fold, P=0.004), Fcgr1a (2.94-fold, P=0.007), Emr1 (2.78-fold, P=0.00002) and Rbbp7 (2.68-fold, P=0.0051) (table 2). Seven of the differentially expressed genes were confirmed with qPCR (figure 2).

The differentially expressed genes grouped into specific “disease categories”, “cellular functions” and “gene networks”

Analyses of the 310 differentially expressed genes revealed an over-representation of ten disease categories and/or molecular and cellular functions as detected by the IPA pathway analyses (table 3). These categories included cancer (89 genes), cell death (68 genes), reproductive system disease (this category predominantly included endometriosis and genes implicated in cancer of the reproductive system or breast) (57 genes), inflammatory disease (53 genes), hematological disease (37 genes) and others (table 3).

The IPA network/pathway detection analyses identified seven additional functional groups over-represented among the differentially expressed genes (supplemental table 1). These included “Embryonic Development, Tissue Development, Cell Death” (27 genes), “Inflammatory Response, Connective Tissue Disorders, Tissue Morphology” (20 genes) and “Cell Cycle, Connective Tissue Development and Function, Organ Morphology” (19 genes), all with significant scores (supplemental table 1).

Inflammatory Cytokines, Chemokines and related genes

We used three approaches to identify differentially expressed cytokines, chemokines and other known inflammatory mediators or receptors: a) genes expressed by all 18 synovial samples and meeting the criteria for significance described in the Methods section ($P \leq 0.01$ plus ≥1.5-fold-difference; b) genes expressed by only one of the strains and not the other; c) genes predominantly expressed in one strain and not in the other. Using these combined parameters we identify 21 cytokines, chemokines, receptors or related genes of relevance (table 4).

Inflammatory mediators and receptors up-regulated in DA—IL-6 was preferentially expressed in 92% of DA (n=11) and only in 33% (n=2) congenic’s synovial tissues (table 4). IL-11 was expressed in significantly increased levels in DA, compared with congenics (1.66-fold, P=0.007). These two pleiotropic cytokines are also expressed in increased levels in RA synovium, and are known to activate NFκB and osteoclasts, and to increase cell tolerance to oxidative stress, all phenomena that take place in the arthritic synovial tissues [8-11].

Cxcl10, Ccl12, and Ccl21b among others, were preferentially expressed, or expressed in significantly increased levels in DA, compared with congenics. Chemokine receptors Ccr6, Cx3cr1 and Cxcr3, as well as cytokine receptors, IL-1rl1 (IL-33r) and IL-3ra were also up-

regulated in the synovial tissues from DA rats. Interestingly, Ccl21b, Ccr6 and Stat4 have been associated with RA susceptibility [12].

Additional important mediators of the inflammatory response up-regulated in DA included Csk, a key signaling gene that regulates T and B cell proliferation, activation, and migration that interacts with the RA susceptibility gene Ptpn22 [2].

Four of the above genes, including IL-6, Stat4, Ccr6 and Cxcl10, have been implicated in the differentiation, activity or chemotaxis of Th17 cells, a cell type central to autoimmune disease pathogenesis. We next looked for the three genes more specifically related to Th17 cells: IL-17A, IL-17F and Rorc (RORgt). The Illumina RatRef microarray does not contain probes for IL-17A and IL-1F. Therefore we used qPCR to study the expression of these two genes. IL-17A was preferentially expressed in DA synovial tissues (93%), compared with DA.ACI(Cia10) congenics (43%), and this difference was statistically significant (p=0.025; Fisher Exact test, table 4). IL-17F was not expressed in either strain's synovial tissues. Rorc (RORgt) expression was not significantly different between the DA and DA.ACI(Cia10) both in the microarray and qPCR (data not shown). Therefore, our results suggest that the Cia10 gene in involved in the regulation of differentiation or synovial tissue homing of Th17 cells.

Inflammatory mediators and receptors up-regulated in DA.ACI(Cia10) congenics—Tgfa, Tgfb2 and the receptor Tgfbr3 were up-regulated or predominantly expressed in DA.ACI(Cia10) congenics, compared with DA (table 4). Tgfb and its receptors are known to suppress immune responses and to induce the differentiation of Treg cells [13]. Several genes up-regulated in DA.ACI(Cia10) congenics are known to interact with, or to be regulated by Tgfb, further suggesting a role for this gene in mediating protection in the congenics (figure 3).

The Vdr gene was predominantly expressed in congenics (80%) compared with DA (33%) (table 4). IPA pathway and gene interaction analyses revealed that several of the most significantly differentially expressed genes were induced by or interacted with the Vdr, including Tgfb2 (figure 3). The Vdr is known to inhibit the expression of cytokines like IL-6, which is in agreement with our observations of an inverse correlation between Vdr-Tgfb2 and IL-6.

Ccl11 (Eotaxin) expression was increased in the protected DA.ACI(Cia10) congenics (1.93-fold, P=0.003). Interestingly, Ccl11 levels have been shown to correlate with reduced radiographic damage in RA via yet unknown mechanisms [14]. Ccl11 is a chemoattractant to eosinophils and Th2 cells, and modulates monocyte activity.

Genes implicated in cancer and cancer-related phenotypes accounted for the greatest percentage of the differentially expressed genes

89 genes of the 310 (28.7%) differentially expressed genes are implicated in cancer and cancer-related phenotypes. These included oncogenes, cell-cycle regulators, tumor-suppressor genes, proteases and others. The 27 genes with the strongest literature support for a role in cancer are listed on table 5. Among the cancer-favoring genes up-regulated in DA

there were eight oncogenes or tumorigenesis genes, including Rbm3, Ikbke, Rcl, Kras, four cell proliferation enhancing genes (Mcts1, Aplnr, Ppap2a, and Ier3) and three invasion-associated genes (Ctsd, Cd53 and Loxl1). Two additional mediators of cancer invasion and metastasis, Cxcl10 and Cxcr3, were preferentially expressed by DA synovial tissues [DA=12 (100%), DA.ACI(Cia10)=3 (50%)] (table 4). IL-11, which as described above was expressed in increased levels in DA (table 4), has also been implicated in the pathogenesis of carcinomas [15]. Histone deacetylases (Hdac1, Hdac2 and Rbbp7) had increased expression in DA, and are also commonly up-regulated in cancers and considered to regulate cell differentiation and proliferation [16].

Nine of the genes up-regulated in DA.ACI(Cia10) are known to suppress a cancer phenotype. Specifically, four of these genes (Pawr, Per1, Igfbp6 and Thbd) negatively regulate cell proliferation and growth, and three genes (Rgs4, Nov and Tgfbr3) negatively regulate cell invasion (table 4). Matrix metalloproteinases (MMPs) are known to increase invasion of cancer and synovial cells and are key mediators of cartilage and joint destruction in RA. Timp2 (2.15-fold, P=0.0024) and Reck (2.14-fold, P=0.005), two MMP inhibitors, were also significantly up-regulated in DA.ACI(Cia10). Four of the cancer-related genes (Cd53, Nov, Reck and Timp2) were confirmed with qPCR (figure 2). Therefore, our results suggest a general increased expression of genes known to favor cell proliferation and invasion in DA, while the congenics has an increased expression of anti-proliferation and anti-invasion/anti-destruction (MMP inhibitors) genes. These observations provide new insight into the molecular processes regulating the reduced synovial hyperplasia and reduced cartilage and joint damage that we had previously reported on DA.ACI(Cia10) congenics compared with DA.

Apoptosis and Cell Survival genes are over-represented among the differentially expressed genes

68 cell death regulatory genes (table 3), including 43 apoptosis genes (13.9% of all differentially expressed) had a significantly different expression between DA and DA.ACI(Cia10) (supplemental table 2). However, a similar number of pro-apoptosis [DA=11 and DA.ACI(Cia10)=8] and anti-apoptosis [DA=12 and DA.ACI(Cia10)=10] genes was up-regulated in DA and in DA.ACI(Cia10). Therefore, while several apoptosis genes were differentially expressed, there was not a clear suggestion of an increased or reduced apoptotic gene expression pattern in either strain.

Anti-oxidant genes are increased in DA.ACI(Cia10)

Genes with anti-oxidant properties (Cat, Mt3, Nfe2l1, and Sqstm1), including Gstm1, which has been associated with disease severity and articular damage in RA [17], were amongst the most significantly up-regulated genes in DA.ACI(Cia10) (figure 3 and table 4).

NFκB pathway interacting genes are differentially regulated between DA and DA.ACI(Cia10)

Ten of the differentially expressed genes are known to interact with the NFκB pathway (supplemental table 3). These included three NFκB activators (Ikbke, Ccl21b and Ikbkap) and one inhibitor (Ucp2) up-regulated in DA. As described above, IL-6 and IL-11, which are

known to activate NFκB, were also expressed in increased levels, or preferentially expressed in DA.

Among the genes up-regulated in DA.ACI(Cia10) congenics, there were those that reduce activation (Cat) and transcription (Pawr and Thbd) of NFκB. Additionally, several genes up-regulated in DA.ACI(Cia10) congenics have been reported to interfere with the NFκB pathway (Supplemental figure 1). Therefore, we looked for 244 NFκB/C-rel target genes previously reported by others [18] and determined that 78 were expressed by all of our samples (32%). Only one of these genes (Acs14) was differentially expressed (2.6-fold up-regulated in DA.ACI(Cia10), P=0.003), suggesting that NFκB was not differentially activated between the two strains.

An *in vitro* NFκB luciferase reporter assay performed in cultured FLS obtained from DA and DA.ACI(Cia10) rat synovial tissues and stimulated with IL-1β did not detect any significant difference in NFκB activity between the two strains, in agreement with the lack on an NFκB expression signature (supplemental figure 1).

Differentially expressed genes located within the DA.ACI(Cia10) interval on chromosome 2

204 of the 7,593 genes expressed by all samples were contained within the Cia10 QTL region on rat chromosome 2. Sixteen of these 204 (7.8%) genes were differentially expressed between DA and DA.ACI(Cia10) congenics, which was more than the 76 (1%) that would have been expected by chance (P=0.018, Chi-square test). Seven of these sixteen genes were expressed in increased levels in DA.ACI(Cia10) and included Pde5a, Palmd, Tspan2, Nes, Adamtsl4 and two antioxidant genes Gstm1 (2.58–fold, P=0.0029) and Gstm2 (2.70–fold, P=0.0028; table 6).

Nine of the above 16 differentially expressed genes were up-regulated in DA, and conversely down-regulated in DA.ACI(Cia10) congenics, and included Cd53, Fcgr1a, Shc1, Tnfaip8l2, gene similar to Gapdh, Sass6, Hist2h2ac, Ncu-g1 and gene similar to Nrf2 (table 6). Cd53 (3.28-fold, P=0.004) and Fcgr1a (2.94-fold, P=0.007) were amongst the most significantly differentially expressed genes.

The differential expression of Cd53, Fcgr1a and Gstm1 was confirmed using qPCR (figure 2). Thirteen of the differentially expressed genes located within the DA.ACI(Cia10) interval are located in the same cytogenetic band on chromosome 2 (2q34) suggesting the possibility that there could be a polymorphism within the region affecting mRNA transcription or stability of multiple genes.

None of the genes contained within the Cia10 interval were preferentially expressed in one strain versus the other.

miRNAs located within the Cia10 interval contain no sequencing polymorphisms

It was hypothesized that part of the differential gene expression pattern could be explained by polymorphisms in a miRNA located within the Cia10 interval. The polymorphism could lead to an alteration of the miRNA expression or function and modulation of target genes. Eight miRNAs are predicted to reside within the Cia10 interval on rat chromosome 2

(miR9-1, miR15b, miR16, miR92b, miR137, miR186, miR190b and miR760). While seven of these microRNAs had differentially expressed targets, none of them had more targets genes than would have been expected by chance (mir9-1: n=11, mir15b: n=16, mir16: n=16, mir92b: n=9, mir137: n=9, mir186: n=5, mir760: n=6). For further confirmation, we sequenced these seven miRNAs and their surrounding DNA (500-1000bp), which contains regulatory regions. However, no polymorphisms were detected between DA and ACI.

DISCUSSION

Increased disease severity and articular damage are associated with increased risk for disability, deformities and reduced life expectancy in patients with RA [19, 20]. Yet, the genes that regulate disease severity and articular damage remain largely unknown. We have been interested in the identification of these genes and consider that they have great potential to generate new and perhaps better targets for therapies aimed at preserving joint integrity and function. In the present study we used synovial tissues from DA rats, which develop severe arthritis (PIA) with pronounced synovial hyperplasia and cartilage and bone destruction, and synovial tissues from arthritis-protected and non-erosive DA.ACI(Cia10) congenics. These two strains are genetically identical except for the presence of ACI alleles at the Cia10 interval, underscoring the magnitude of the effect of this single locus on clinical disease, histologic joint damage [7] and gene expression.

DA.ACI(Cia10) and DA had significant differences in the expression of inflammatory mediators. That difference was not broad, but instead limited to a very specific set of genes, most of which have been involved in the regulation of cartilage and bone erosions, and articular damage. IL-6 and IL-11, which belong to the IL-6 family of genes and signal through gp130, were among the cytokines with significantly reduced expression in DA.ACI(Cia10) congenics. Both IL-6 and IL-11 are expressed in increased levels in RA synovium, and while IL-6 has been clearly implicated in arthritis pathogenesis and joint damage [8, 9], the literature on IL-11 is contradictory [10, 11]. The chemokines Ccl12, Ccl21b and Cxcl10, and chemokine receptors Ccr6, Cx3cr1 and Cxcr3 were also expressed in reduced levels in congenics. Ccl12 is chemotactic for Ccr2-expressing monocytes and neutrophils, which have a key role in the development of synovial hyperplasia and joint erosions and damage [21]. Ccl21b is chemotactic for Ccr7-expressing T and B cells, stimulates FLS, and has been implicated in the organization of germinal center-like structures in synovial tissues of RA [22, 23]. Cx3cr1 is expressed by myeloid cells, and its blockade ameliorates rodent arthritis and reduces articular damage [24]. Cxcl10 and its receptor Cxcr3 was predominantly expressed in DA, and these genes mediate T cell and mast cell chemotaxis, and increase FLS invasion in an autocrine and paracrine manner (Laragione et al. unpublished observations). Furthermore, blockade of Cxcr3 ameliorates adjuvant-induced arthritis in rats, also reducing joint erosion and damage [25].

Synovial tissues from DA.ACI(Cia10) congenics had increased expression of Tgfb2 and Ccl11. Tgfbr3 was also preferentially expressed in congenics' synovial tissues. Tgfb is considered a suppressor of T cell responses and has a central role in the differentiation of Treg cells. Furthermore, treatment with Tgfb ameliorates autoimmune arthritis in rodents while anti-Tgfb antibodies exacerbate disease [26]. Ccl11, also known as Eotaxin-1, is an

eosinophil chemoattractant expressed by T-cells and fibroblasts. Increased serum levels of Ccl11 have been associated with reduced radiographic damage progression in early RA [14]. Lastly, the Vdr was predominantly expressed in the synovial tissues of congenics. The Vdr has known IL-6-suppressive [27], Th17 differentiation-inhibitory properties [28], and Tgfb-inducing activities (figure 3), which matches the scenario identified in this study. Vdr agonists also reduced arthritis severity [29]. Together, our findings suggest that Cia10 directly or indirectly regulates the expression of genes implicated in arthritis severity and joint damage, with increased levels or preferential expression of pro-inflammatory and joint damage-favoring genes in DA, while the opposite takes place in congenics.

While IL-23, IL-17-F and RORgt (Rorc) were not differentially expressed in synovial tissues collected on day 21, four of the genes up-regulated or preferentially expressed in DA, and conversely down-regulated in DA.ACI(Cia10) congenics, suggest an increased presence of IL-17 producing or inducing cells: a) IL-17A was expressed in nearly all DA samples (93%), but only in 43% of congenics; b) Ccr6 is a cell surface marker of Th17 T cells [30]; c) Stat4 mediates IL-23r signaling, which is required for the generation of Th17 cells; and d) Cxcr3 is expressed by mast cells, which have been considered the main source of IL-17 in the synovial tissue [31]. Furthermore, DA synovial tissues preferentially expressed IL-6, which is a key cytokine for differentiation of Th17 cells, but lacked Vdr expression, which is an inhibitor of Th17 differentiation. These observations raise the possibility that Cia10 could be a new gene controlling arthritis severity indirectly via the regulation of the differentiation and/or homing of Th17 cells to the joint.

Four of the genes with the most significantly reduced expression in DA.ACI(Cia10) congenics are either associated with genetic susceptibility to RA (Ccr6, Ccl21b, Stat4) [12, 32], or as in the case of Csk, directly interact and regulate the activity of a RA susceptibility gene (PTPN22) [2]. Furthermore, Gstm1 null alleles have been associated with RA severity and radiographic erosive damage [17], and DA synovial tissues did in fact have significantly reduced expression compared with DA.ACI(Cia10). However, sequencing of DA Gstm1 gene revealed no deletions, insertions or significant sequence variants that might explain the reduced expression (data not shown). Taken together, our results suggest that the Cia10 gene could interact with RA susceptibility genes to regulate their expression and perhaps function, raising the possibility of potential epistatic interactions.

NFκB is a central regulator of synovial hyperplasia and articular damage [33, 34], and several RA susceptibility genes are involved in this pathway [35]. A subset of the differentially expressed genes in the present study, including IL-6, IL-11 and Ccl21b, are known activators of NFκB, and NFκB-interacting genes were differentially expressed, raising the possibility that Cia10 might be involved in the regulation of NFκB activity. However, we were not able to detect a differentially expressed NFκB transcriptional signature [18]. For further confirmation a NFκB luciferase reporter assay was studied in FLS from DA and DA.ACI(Cia10) congenics stimulated with IL-1β, and did not detect any significant difference in transcriptional activity. Therefore, our results suggest that Cia10 does not have a major effect on the regulation of the NFκB pathway.

There are several similarities between the pathogenesis of RA synovial hyperplasia, its invasion and destruction of cartilage and bone, and the behavior of cancers [36-38]. Previous studies from our group and others have identified increased expression of cancer-related genes in FLS from arthritic rats and from patients with RA [39-41]. In the present study one of the predominant differentially expressed groups was related to cancer and cancer phenotypes. Genes involved in oncogenesis (Kras, Ikbke, St14), cell proliferation (Cdc25a, Ier3, Ppap2a), cancer invasion (Cd53, Ctsd, Cxcl10, Cxcr3, Loxl1) [42-46] and histone deacetylation (Hdac1, Hdac2 and Rbbp7) [16] were expressed in increased levels in DA and in reduced levels in congenics.

On the contrary of the cancer-favoring genes up-regulated in DA, genes up-regulated in DA.ACI(Cia10) were protective against a cancer phenotypes. For instance, Nov, Rgs4 and Tgfbr3, which are inhibitors of cancer cell migration and invasion [47-49], were expressed in increased levels in DA.ACI(Cia10). Two MMP inhibitors (Timp2 and Reck) were up-regulated in DA.ACI(Cia10) and down-regulated in DA. Reck negatively regulates cancer cell invasion, metastasis and angiogenesis in cancer, and its expression is reduced in cancer cells [50, 51]. Like in cancers and in DA synovium, Reck is expressed in reduced levels in RA synovial tissues, compared with OA controls [52]. Timp2 is a negative regulator of MMPs, angiogenesis, cancer cell growth, invasion and metastasis [53, 54]. Taken together, our results show that the presence of DA alleles at Cia10 increases the expression of genes favoring cancer development, proliferation and invasion, while the opposite is seen in the presence of ACI alleles. These observations suggest that these Cia10-regulated cancer genes could be involved in the regulation of synovial hyperplasia and pannus invasion and destruction of cartilage and bone.

Survival and apoptosis abnormalities have been described in cancer, in arthritic synovial tissues and in autoimmunity. We considered that the synovial hyperplasia in DA, and the lack of in DA.ACI(Cia10) could be attributed to differences in expression of apoptosis genes. While many apoptosis and cell survival genes were differentially expressed, there was not a clear bias towards having increased numbers of pro-apoptosis versus anti-apoptosis genes in either strain.

Thirteen of the sixteen differentially expressed genes located within the DA.ACI(Cia10) interval map to the same cytogenetic band on chromosome 2 (2q34) raising the possibility that a polymorphism within the region could affect a regulatory element acting in *cis* to influence the transcription of these closely located genes. While *cis*-acting transcription factor binding site polymorphisms have been a rare explanation for autoimmunity or other complex traits, it is certainly a possibility worth considering.

We also considered that a polymorphism in a microRNA located within the Cia10 interval might explain part of the differences in synovial gene expression. The Cia10 interval contains eight predicted microRNAs but sequencing these microRNAs revealed no polymorphisms. Additionally, analyses of the expression of predicted microRNA targets revealed no significant differences between the strains, making the microRNA hypothesis unlikely to explain the Cia10 effect.

In conclusion, we have determined that Cia10 regulates the expression of a unique set of inflammatory mediators, including markers of Th17 cells, IL-6, IL-17A and IL-11, oxidative stress regulators and a cancer-associated gene expression signature. These observations suggest a mechanism of action for the Cia10 gene, and several new potential prognostic biomarkers and targets for therapies aimed at reducing disease severity and joint damage in RA.

MATERIAL AND METHODS

Rats and generation of DA.ACI(Cia10) congenic strain

DA (arthritis-susceptible) and ACI (arthritis resistant) rats were originally purchased from Harlan Sprague Dawley (Indianapolis, IN). DA.ACI(Cia10) congenic rats were generated as previously described [7]. Briefly, a 52.6 Mbp region from the ACI strain containing the Cia10 interval on chromosome 2 (Figure 1A) was introgressed into DA through genotype-guided breeding. This strategy selected for ACI alleles at the Cia10 interval while at the same time excluding donor genome contamination at other loci known to regulate arthritis severity [6, 55]. After five backcrosses offspring heterozygous at the Cia10 interval were intercrossed to generate Cia10 homozygotes for ACI alleles (DA.ACI(Cia10)) to be used in experiments. The experimental protocol was reviewed and approved by the FIMR Institutional Animal Care and Use Committee.

Induction of PIA and arthritis scoring

Male DA (n=12) and DA.ACI(Cia10) (n=6) congenic rats 8-12 week-old were anesthetized and injected intradermally at the base of the tail with 150 μl of pristane (MP Bio, Solon, OH) divided into two injection sites (day 0) [7, 56]. Arthritis severity was assessed using a previously described 80-point scoring system that uses the sum of scores to generate an arthritis severity index (ASI) [7]. Ankle synovial tissues were collected for analysis 21 days post-induction of PIA.

RNA extraction

Synovial tissue was homogenized in lysis buffer using a rotor-stator homogenizer and total RNA was extracted using RNeasy (Qiagen, Valencia, CA) and including a DNase treatment step, according to manufacturer's instructions. RNA was quantified and assessed for purity using the NanoDrop spectrophotometer (Rockland, DE), and RNA integrity was verified using the BioAnalyzer 2100 (Agilent, Palo Alto, CA).

Microarray experiments

The microarray protocol was previously described [40]. Briefly, 200 ng of total RNA was amplified and biotinylated using the TotalPrep labeling kit (Ambion, Austin, TX). RNA samples were hybridized to the RatRef-12 Expression BeadChip (Illumina, San Diego, CA), which contains 22,524 probes covering 21,922 rat genes selected primarily from the NCBI RefSeq database (Release 16). Hybridization was carried out in Illumina IntelliHyb chambers, followed by washing and staining with Cy3-streptavidin. The array was scanned on a high-resolution Illumina BeadArray reader using a two-channel 0.8 μm resolution

confocal laser scanner. Protocols were previously optimized for use with the Illumina Whole-Genome Expression platform.

Microarray data analysis

Illumina Bead studio software (Version 2.0) was used to extract data, which was normalized using the cubic spline algorithm. In order to reduce the false positive rate, only genes expressed by all 18 synovial tissues were used for analyses. Mean intensity values for genes detected in all 18 samples were Log_2-transformed and expression data from DA and DA.ACI(Cia10) compared using the t-test. Fold-differences were calculated by elevating 2 to the difference between the means of log_2-transformed DA and log_2-transformed DA.ACI(Cia10) values [$2^{(\text{mean } Log_2 DA - \text{mean } Log_2 \text{ Cia10})}$], and vice-versa for DA.ACI(Cia10) fold-differences. Genes with a P-value ≤0.01 (t-test) and a fold-difference ≥1.5 between DA and DA.ACI(Cia10) were considered significantly different. Ingenuity IPA 5.5.1 program (Ingenuity, Redwood City, CA, USA) and publicly available databases such as Pubmed and Online Mendelian Inheritance in Man (OMIM) were used for pathway discovery and analysis. Enrichment for biological functions and disease groups was determined with the IPA software and calculated using the Fisher's exact test with a cutoff p-value of ≤0.05, using the Benjamini-Hochberg correction.

We also looked for genes **a)** expressed in synovial tissues of one strain and not by the other, or **b)** predominantly in one strain and not in the other, since those could represent even more significant differences relevant to disease pathogenesis. None of the genes met criteria "**a**" (expressed only by one strain and not by the other), but several met criteria "**b**".

cDNA synthesis and quantitative PCR (qPCR)

cDNA was synthesized from the same RNA samples hybridized to the RatRef-12 Expression BeadChip. 2μl of total RNA was used for cDNA synthesis using the Superscript III kit (Invitrogen Carlsbad, CA). cDNA was diluted 1:10 for qPCR. Rat specific primers and probes were designed using the Universal ProbeLibrary (Roche, Indianapolis, IN) (table 1). Gapdh was used as an internal control and all samples were run in duplicate. The average threshold cycle (Ct) values were used to analyze relative gene expression of Cd53, Fcgr1a, Gp49b, Timp2 Reck, Nov and Gstm1. Probes were used at a final concentration of 100nM, 5' ends were labeled with FAM and 3' ends with TAMRA. Primers were used at 200 nM with Eurogentec qPCR mastermix (Eurogentec, San Diego, CA). Reactions were run on an ABI 7700 qPCR thermocycler at 50°C for 2 minutes, 95°C for 10 minutes, and 45 cycles of 95°C for 0.15 minutes and 60°C for 1 minute. Relative expression of genes was normalized to Gapdh in each sample (ΔCt), and ΔCt values were used for t-test analysis. qPCR fold-differences were calculated using the $2^{-\Delta\Delta Ct}$ method [57].

microRNA analyses

The miRBase.org database was used to identify the microRNAs located within the Cia10 interval, and TargetScan.org was used to predict their targets. The eight microRNAs contained within the Cia10 and with differentially expressed predicted targets were sequenced as described below.

Sequencing

Splenic genomic DNA (gDNA) was extracted using DNeasy Blood and Tissue Kit (Qiagen). Primers were designed using the Whitehead Institute Primer3 website (http://frodo.wi.mit.edu/primer3/input.htm) to amplify 500-1000bp regions surrounding eight known microRNA sequences located within the Cia10 interval (table 1). PCR products were sequenced and analysed with the DNASTAR sequencing analysis software (Madison, WI).

NFκB luciferase reporter assay

Synovial tissues were collected from a different group of DA and from DA.ACI(Cia10) joints 21 days after the induction of PIA for fibroblast-like synoviocyte (FLS) isolation. FLS were isolated as previously described [40]. Cells from passage four or greater (>95% FLS purity) were used for the experiments. 4.6×10^4 cells per cell line per strain were co-transfected with a NFκB luciferase reporter plasmid (NFκB binding sequences were originally cloned into a Promega pGL2 construct at Dr. L. Klampfer's laboratory, Albert Einstein College of Medicine, Bronx, NY) and a Renilla internal control plasmid (Promega, Madison, WI) using Lipofectamine 2000 (Invitrogen). Control cells were transfected with a luciferase plasmid without a promoter. Following transfections cells were cultured overnight on FBS-free medium, followed by stimulation with IL-1β 10ng/ml for 48h. The NFκB reporter activity was measured with the TD 20/20 Luminometer (Turner Designs, Sunny vale, CA). Arbitrary units of the Renilla reporter were used for internal normalization.

Statistical Analysis

The t-test was used to compare the expression of genes expressed by all samples. Chi-squared and Fisher exact tests were performed using SigmaStat 3.0 (SPSS, Chicago, IL).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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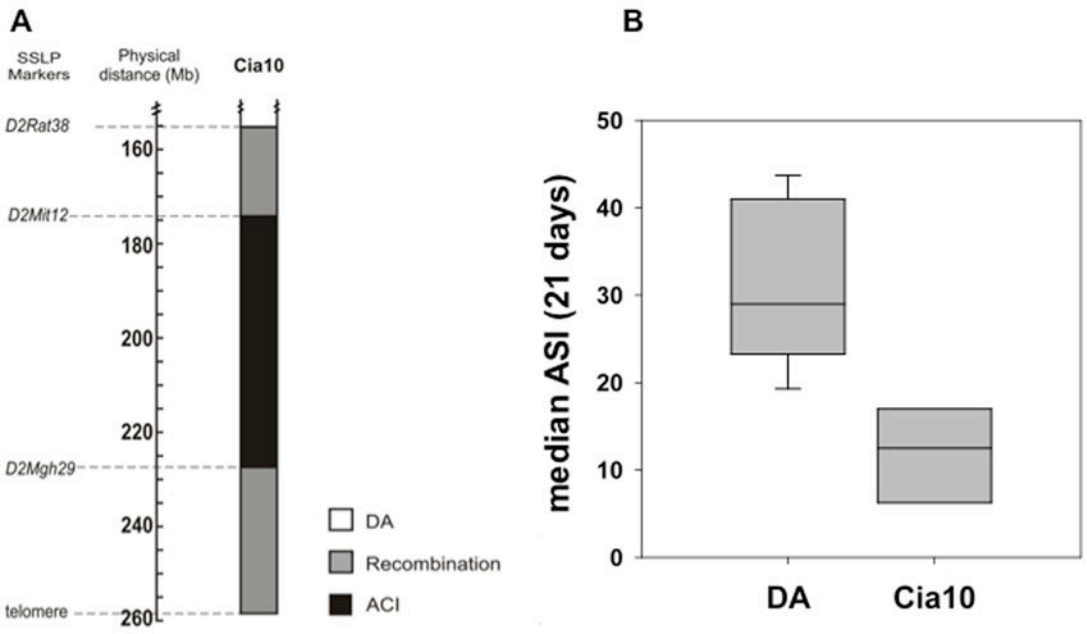

Figure 1. A map of the Cia10 interval in DA.ACI(Cia10) congenics and arthritis severity index (ASI) scores

A. Map of the DA.ACI(Cia10) congenic interval on rat chromosome 2 showing DA alleles (white), ACI alleles (black) and recombination interval (grey). Key SSLP markers used in the congenic breeding are shown (Mb=position in megabases on the rat genome assembly v. 3.4). **B.** Cummulative median ASI scores of DA (ASI=30.4) compared with DA.ACI(Cia10) (ASI=12.3) during 21 days following PIA induction (P=0.002 Mann-Whitney test) (DA n=12 and DA.ACI(Cia10) n=6). Boxes represent the 25%-75% confidence interval, and error bars are the 5%-95% confidence interval.

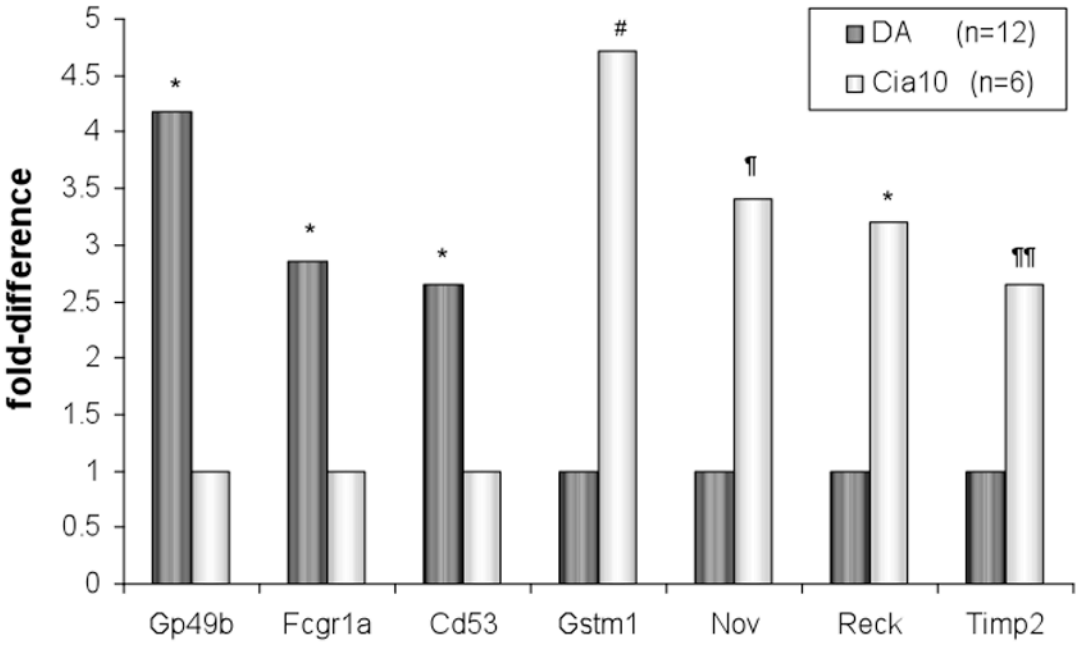

Figure 2. qPCR confirmation of the microarray results

Three of the genes most significantly up-regulated in DA (Gp49b, Cd53 and Fcrg1) and four of the most up-regulated genes in DA.ACI(Cia10) (Timp2, Reck, Gstm1 and Nov) were tested with qPCR. [DA n=12 and DA.ACI(Cia10) n=6; Reck was tested in 7 DA and 4 DA.ACI(Cia10)]. * $P<0.004$; # $P=0.016$; ¶ $P=0.089$; ¶¶ $P=0.25$ (t-test using ΔCt values).

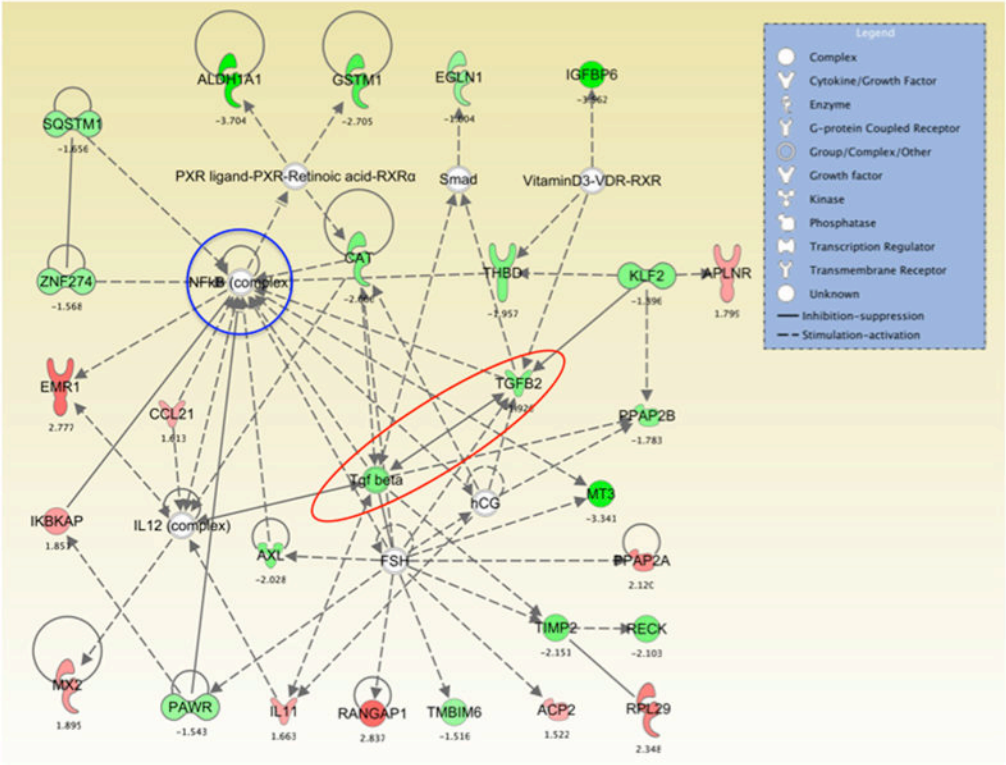

Figure 3. Network analyses of differentially expressed genes suggest a central role for Tgfβ and NFκB

Ingenuity pathway analyses determined that several of the most significantly differentially expressed, or preferentially expressed genes interact with, induce or are induced by Tgfβ (red elipse) and NFκB (blue circle). These genes included Cat, Igfbp6, Reck, Timp2 and Vdr in the case of Tgfβ, and Ccl21, Emr1, IκBkap, IL-11 and Rangap1 in the case of NFκB.

Table 1

Primers and probes used for microarray results' validation with qPCR, and primers used for microRNA sequencing[§].

Gene Symbol	Accession Number	Forward primer	Reverse primer	Probe number
qPCR				
Cd53	NM_012523.1	GGGACTTCATCCAGTCACAAC	AACCCTGAACATCTGCACCT	4
Fcgr1a	NM_001100836.1	CCTGTGCAGTTAGAAATCTACAGAGA	CTTATTCTGCCACGGGTGA	110
Gapdh	NM_017008	GAACGGGAAGCTCACTGGC	GCATGTCAGATCCACAACGG	Taqman probe
Gstm1	NM_017014.1	TGTTACAACCCCGACTTTGA	TCTTCTCAGGGATGGTCTTCA	106
LOC499078*	XM_001070660.1	GGAGAGCTGGACAGTTGGAA	GGTTACAACCTGGGTCTCTTTG	80
Nov	NM_030868.2	CGGCCTTGTGAGCAAGAG	TTCTTGGTCCGGAGACACTT	60
Reck	NM_001107954.1	AAAAGTTGGACACAATTGCTAAGA	TCGGAGTTGATGAGGGACTC	38
Timp2	NM_021989.2	CTGGACGTTGGAGGAAAGAA	ACAGAGGGTAATGTGCATCTTG	12
Rorc	RGD:1595785	AGCAGAACTGCCCCATTG	TCCCTCTGCTTCTTGGACAT	21
Il17a	NM_001106897	CTTCACCCTGGACTCTGAGC	CCTCAGCGTTGACACAGC	98
Il17f	NM_001015011.1	GGCTCCTGTGAAACAACCAT	TGATGGCAAATCCCAACAT	84
Sequencing				
mir9-1	MI0000838	CAGCTTAGATTCCCGACCTCAGAAC	ATCTTGACCCCCAGTAAAGCTGAAG	---
mir15b/16[#]	MI0000843/MI0000844	TGACGTCCTTCCTAACAGCAACTTC	TGTGTACAAATTAAACCCACGCAAA	---
mir92b	MI0006167	GAGGTATGGGTGGGAAAAGTACCAA	TTAAAAACATTTCGGAGGTGCATGA	---
mir137	MI0000910	AAGCAGAGCAAATACCAAGGAAAGC	ACCACCCGAGGAAAAGAAAACATAA	---
mir186	MI0000931	TTCTAGCATTTGAGGGCAGTTACCA	GGTGCTACAGACAACTGAGGGACAT	---
mir190b	MI0006135	AGAGGAGATGCAAACCCCTAACCTT	AGACAATAGTCTCAGAGCCCCAGGA	---
mir760	MI0006164	CTCTAGAAGTCCAATGCGCTATCCA	AGGAAACTGAGGACCCACTCCTTC	---

[§]microRNA=miR and its accession numbers were obtained from Ensembl and the miRBase;

Table 2

Most significantly differentially expressed genes between DA.ACI(Cia10) congenics and DA.

Gene symbol	Gene name	Accession number	Fold difference	P-value
Up-regulated in DA			**DA/Cia10**	
LOC499078	similar to glycoprotein 49b	GI_62638899-S	5.39	0.0023
Cd53	cluster of differentiation 53	NM_012523.1	3.28	0.0040
RGD1566002	similar to RIKEN cDNA 3110001N18	XM_221003.3	3.09	0.0038
Fcgr1a	Fc fragment of IgG, high affinity Ia, receptor (CD64)	XM_001062370.1	2.94	0.0073
ccdc109b	coiled-coil domain containing 109B	XM_001076863.1	2.89	0.0036
LOC499136	hypothetical gene	XM_574429.1	2.85	0.0014
Rangap1	RAN GTPase activating protein 1	XM_576313.1	2.84	0.0051
RGD1565520	similar to 60S ribosomal protein L7a	XR_009158.1	2.81	0.0025
Emr1	egf-like module containing mucin like hormone receptor	NM_001007557.1	2.78	0.0000
LOC308350	similar to PIRB1	XM_218261.3	2.73	0.0074
RGD1564866	similar to Heterogeneous nuclear ribonucleoprotein A1	XR_007647.1	2.72	0.0038
LOC687849	hypothetical gene	XM_001080339.1	2.69	0.0012
Rbbp7	Retinoblastoma-binding protein 7	NM_031816.1	2.68	0.0052
RGD1563503	similar to ribosomal protein L6	XR_007388.1	2.67	0.0010
RGD1563124	similar to 40S ribosomal protein S20	XM_576204.2	2.66	0.0013
RGD1559935	similar to Selenoprotein H	XR_008210.1	2.66	0.0027
Ppid	peptidylprolyl isomerase D	NM_001004279.1	2.66	0.0003
RGD1560979	hypothetical gene	XR_008629.1	2.65	0.0031
Acsl4	acyl-CoA synthetase long-chain family member 4	NM_053623.1	2.62	0.0033
St14	suppression of tumorigenicity 14	NM_053635.2	2.55	0.0004
Shc1	Src homology 2 domain-containing transforming protein C1	NM_053517.1	2.55	0.0001
LOC306428	similar to Chain A, T13s Mutant Of Bovine 70 Kilodalton Heat Shock Protein	XM_224824.3	2.55	0.0007
LOC316373	similar to high-mobility group (nonhistone chromosomal) protein 1-like 1	XR_007934.1	2.54	0.0064
Fcgr3a	Fc receptor, IgG 3a	NM_207603.1	2.51	0.0021
Rsl1d1	ribosomal L1 domain containing 1	NM_001008876.1	2.50	0.0032
Matk	megakaryocyte-associated tyrosine kinase	NM_021859.2	2.49	0.0044
RGD1559682	similar to peptidylprolyl isomerase A	XM_001075526.1	2.47	0.0042
Fcgr2b	Fc fragment of IgG, low affinity IIb, receptor (CD32)	NM_175756.1	2.45	0.0090
Smc4l1	structural maintenance of chromosomes 4	XM_001066172.1	2.43	0.0059
Tnfaip8l2	Tumor necrosis factor, alpha-induced protein 8-like 2	NM_001014039.1	2.42	0.0095
Psmb9	proteasome subunit beta-1i	NM_012708.1	2.41	0.0002
RGD620382	Nucleoside 2-deoxyribosyltransferase domain containing protein	NM_133525.1	2.38	0.0037
RGD1565238	similar to Gapdh	XM_001081651.1	2.37	0.0097
RGD1565306	similar to 60S ribosomal protein L29	XM_576023.2	2.35	0.0064
Up-regulated in DA.ACI(Cia10)			**Cia10/DA**	
LOC259244	alpha-2u globulin PGCL3	NM_147212.1	5.01	0.0067
Mup4	alpha-2u globulin PGCL3	NM_198784.1	4.88	0.0067

Gene symbol	Gene name	Accession number	Fold difference	P-value
LOC682605	alpha-2u globulin PGCL3	XM_001062261.1	4.32	0.0074
MGC72973	beta-globulin	NM_198776.1	3.71	0.0095
Aldh1a1	aldehyde dehydrogenase 1 family, member A1	NM_022407.3	3.70	0.0090
Rpesp	RPE-spondin	XM_001063197.1	3.56	0.0082
Igfbp6	insulin-like growth factor binding protein 6	NM_013104.2	3.56	0.0068
Ccdc3	colied coil domain containing 3	XM_574081.1	3.44	0.0097
Mt3	metallothionein 3	NM_053968.2	3.34	0.0089
RGD1310507	similar to RIKEN cDNA 1300017J02	XM_236574.4	3.27	0.0072
Fxyd6	FXYD domain-containing ion transport regulator 6	NM_022005.1	3.26	0.0090
Cav2	caveolin 2	NM_131914.2	3.02	0.0058
Fhl1	four and a half LIM domains protein 1	NM_145669.2	3.01	0.0086
Nov	nephroblastoma overexpressed gene	NM_030868.2	2.86	0.0070
Per1	period 1	XM_340822.2	2.78	0.0011
Myh11	myosin, heavy chain 11, smooth muscle	XM_573030.2	2.77	0.0057
Gstm2	glutathione S-transferase, mu 2	NM_177426.1	2.71	0.0028
Klhl23	similar to 60S ribosomal protein L7a	XM_001059088.1	2.60	0.0007
Pcsk1n	proprotein convertase subtilisin/kexin type 1 inhibitor	NM_019279.1	2.58	0.0072
Gstm1	glutathione S-transferase, mu 1	NM_017014.1	2.58	0.0029
Hbb	hemoglobin, beta	NM_033234.1	2.55	0.0063
Etl4	enhancer trap locus 4	XM_001069190.1	2.48	0.0069
Ctnnal1	catenin (cadherin associated protein), alpha-like 1	XM_001059679.1	2.36	0.0077
Sptbn1	spectrin, beta, non-erythrocytic 1	NM_001013130.1	2.24	0.0041
Cyb5r1	cytochrome b5 reductase 1	NM_001013126.1	2.20	0.0010
Timp2	tissue inhibitor of metalloproteinases-2	NM_021989.2	2.15	0.0024
LOC500248	similar to 1300014I06Rik protein	XM_580052.1	2.14	0.0078
Reck	reversion-inducing-cysteine-rich protein with kazal motifs	XM_001070551.1	2.10	0.0050
Cldn22	claudin 22	XM_224843.2	2.10	0.0019
Ddit4	DNA-damage-inducible transcript 4	NM_080906.1	2.09	0.0090
RGD1311307	similar to 1300014I06Rik protein	NM_001025719.1	2.08	0.0074
Cat	catalase	NM_012520.1	2.07	0.0076
Tmem117	transmembrane protein 117	XM_576330.2	2.06	0.0023
Nes	nestin	NM_012987.1	2.06	0.0060
Rgs4	regulator of G-protein signaling 4	NM_017214.1	2.05	0.0079
Hexim1	hexamethylene bis-acetamide inducible 1	XM_573204.1	2.04	0.0022
Axl	AXL receptor tyrosine kinase	NM_001013147.1	2.03	0.0046
Tspan2	tetraspanin-2	NM_022589.1	2.02	0.0057

Table 3

Diseases and cellular functions overrepresented among the differentially expressed genes.

Disease or cellular function	Number of Genes	p-value
Cancer	89	0.04-0.0005
Cell Death	68	0.04-0.00004
Reproductive System Disease*	57	0.04-0.0002
Inflammatory disease	53	0.04-0.0005
Hematological disease	37	0.04-0.0001
Cellular Assembly and Organization	27	0.04-0.0001
Tissue Development	26	0.04-0.0001
Molecular Transport	25	0.04-0.0002
Cellular Function and Maintenance	21	0.04-0.0001
Immunological Disease	19	0.04-0.0002

* predominantly reproductive and breast cancer phenotypes and endometriosis.

Table 4

Cytokine and chemokine-related genes, and oxidative stress inhibitors differentially or predominantly expressed in DA.ACI(Cia10) or DA.

Gene symbol	Gene name	Accession number	Expressed in all samples		Preferential strain expression		
			Fold-difference	*P-value***	*DA positive (%)*	*Cia10 positive (%)*	*P-value***
Genes up-regulated in DA							
Ccl21b§	chemokine (C-C motif) ligand 21b	NM_001008513.1	1.61	0.002			
Csk	c-src tyrosine kinase	XM_236290.3	2.02	0.002			
IL-11	interleukin-11	XM_346531.2	1.66	0.007			
IL-3ra	interleukin-3 receptor subunit alpha	NM_139260.1	1.52	0.009			
Genes predominantly expressed in DA							
Ccl12	chemokine (C-C motif) ligand 12	XM_213425.2			11 (92)	2 (33)	0.021
Ccr6§	chemokine (C-C motif) receptor	NM_001013145.1			12 (100)	3 (50)	0.024
Cx3cr1	chemokine (C-X3-C motif) receptor 1	NM_133534.1			10 (83)	1 (16)	0.012
Cxcl10	chemokine (C-X-C motif) ligand 10	NM_139089.1			12 (100)	3 (50)	0.024
Cxcr3	chemokine (C-X-C motif) receptor 3	NM_053415.1			12 (100)	3 (50)	0.024
Ifi47	interferon gamma inducible protein 47	NM_172019.1			9 (75)	0 (0)	0.009
IL-10	interleukin 10	NM_012854.1			8 (67)	0 (0)	0.012
IL-1rl1 (IL-33R)	interleukin 1 receptor-like 1	NM_013037.1			12 (100)	2 (33)	0.005
IL-6	interleukin-6	NM_012589.1			11 (92)	2 (33)	0.021
IL-17A	**interleukin-17A**	**NM_001106897**			**13 (93)**	**3 (43)**	**0.025**
Stat4§	signal transducer and activator of transcription 4	NM_001012226.1			11 (92)	2 (33)	0.021
Genes up-regulated in DA.ACI(Cia10)							
Ccl11	chemokine (C-C motif) ligand 11 (eotaxin)	NM_019205.1	1.93	0.003			
Tgfa	transforming growth factor alpha	NM_012671.1	4.20	0.006			
Tgfb2	transforming growth factor beta 2	NM_031131.1	1.93	0.0001			
Tnfsf12	tumor necrosis factor ligand superfamily member 12 (TWEAK)	NM_001001513.2	2.21	0.003			
Cat*	catalase	NM_012520.1	2.07	0.008			
Gstm1*#	glutathione S-transferase mu 1	NM_017014.1	2.71	0.003			
Mt3*	Metallothionein-3; growth inhibitory factor (GIF)	NM_053968.2	3.34	0.009			
Nfe2l1*	nuclear factor, erythroid derived 2,-like	XM_340886.3	1.76	0.009			

Gene symbol	Gene name	Accession number	Expressed in all samples		Preferential strain expression		
			Fold-difference	*P-value***	*DA positive (%)*	*Cia10 positive (%)*	*P-value***
Sqstm1*	sequestosome 1	NM_175843.3	1.66	0.002			
Genes predominantly expressed in DA.ACI(Cia10)							
Tgfbr3	transforming growth factor, beta receptor III	NM_017256.1			6 (50)	6 (100)	0.053
Vdr	vitamin D (1,25- dihydroxyvitamin D3) receptor	NM_017058.1			4 (33)	4 (80)	0.321

§ genes associated with genetic susceptibility in rheumatoid arthritis;

gene associated with disease severity in rheumatoid arthritis. Grey box: gene implicated in Th17 cell responses.

* Inhibitors of oxidative stress;

** Genes expressed in all samples were compared with t-test, and preferecial strain expression with Fisher Exact Test.

IL-17A results are shown in BOLD and reflect qPCR data of 14 DA and 7 DA.ACI(Cia10) rats.

Table 5

Differentially expressed genes related to cancer*

Gene symbol	Accession number	Gene name	Fold difference	P-value
Up-regulated in DA				
Oncogenes and tumorigenesis			**DA/Cia10**	
Rcl (C6ORF108)	NM_133525.1	Nucleoside 2-deoxyribosyltransferase domain containing protein (RGD620382)	2.38	0.004
Kras	NM_031515.1	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	1.93	0.005
Rbm3	XM_001063211.1	RNA binding motif (RNP1, RRM) protein 3	1.57	0.009
Ikbke	XM_001058036.1	Inhibitor of nuclear factor kappa-B kinase subunit epsilon	1.51	0.007
St14	NM_053635.2	suppression of tumorigenicity 14	2.55	0.0004
Cdc25a	NM_133571.1	cell division cycle 25 homolog A	1.67	0.007
Hdac1	XM_216349.3	histone deacetylase 1	1.52	0.006
Hdac2	XM_342149.3	histone deacetylase 2	1.53	0.0007
Cell proliferation				
Mcts1	XM_001064848.1	malignant T cell amplified sequence 1	2.20	0.001
Ppap2a	NM_022538.1	phosphatidic acid phosphatase type 2A	2.12	0.002
Ier3	NM_212505.1	immediate early response 3	1.93	0.008
Aplnr	NM_031349.2	apelin receptor	1.80	0.009
Cell invasion				
Cd53	NM_012523.1	cluster of differentiation 53	3.28	0.004
Loxl1	NM_001012125.1	lysyl oxidase-like 1	1.92	0.005
Ctsd	NM_134334.2	cathepsin D	2.27	0.001
Histone deacetylation				
Rbbp7	NM_031816.1	retinoblastoma binding protein 7	2.68	0.005
Hdac2	XM_342149.3	histone deacetylase 2	1.53	0.001
Hdac1	XM_216349.3	histone deacetylase 1	1.52	0.007
Up-regulated in DA.ACI(Cia10)				
Inhibitors of cell proliferation and growth			**Cia10/DA**	
Igfbp6	NM_013104.2	insulin-like growth factor binding protein 6	3.56	0.007
Per1	XM_340822.2	period 1	2.78	0.001
Thbd	NM_031771.2	thrombomodulin	1.96	0.002
Pawr	NM_033485.2	PRKC, apoptosis, WT1, regulator	1.54	0.005
Inhibitors of invasion				
Nov	NM_030868.2	nephroblastoma overexpressed gene	2.86	0.007
Rgs4	NM_017214.1	regulator of G-protein signaling 4	2.05	0.008
Tgfbr3#	NM_017256.1	transforming growth factor, beta receptor III	-	-
Inhibitors of MMPs				
Timp2	NM_021989.2	tissue metallopeptidase inhibitor 2	2.15	0.002
Reck	XM_001070551.1	reversion-inducing-cysteine-rich protein with kazal motifs	2.10	0.005

*Some of these genes have more than a single cancer-related function.

[#]Tgfbr3 was expressed by 6 (50%) DA and 6 (100%) DA.ACI(Cia10) synovial tissues. Tgfbr3 is also a suppressor of cancer growth and cell proliferation.

Table 6

Differentially expressed genes expressed within the confirmed Cia10 region on chromosome 2.

Gene symbol	Accession number	Gene name	Cytoband	Fold difference	P-value
Genes up-regulated in DA				**DA/Cia10**	
Cd53	NM_012523.1	cluster of differentiation 53	2q34	3.28	0.0040
Fcgr1	XM_001062370.1	Fc receptor, IgG, high affinity I	2q34	2.94	0.0073
Shc1	NM_053517.1	Src homology 2 domain-containing transforming protein C1	2q34	2.55	0.0001
Tnfaip8l2	NM_001014039.1	Tumor necrosis factor, alpha-induced protein 8-like 2	2q34	2.42	0.0095
RGD1560825	XM_227659.3	similar to glyceraldehyde-3-phosphate dehydrogenase	2q42	1.99	0.0088
Sass6	XM_227619.4	spindle assembly 6 homolog (C. elegans)	2q41	1.73	0.0058
Hist2h2ac	XM_574997.1	histone cluster 2, H2ac	2q34	1.57	0.0031
RGD1303130	NM_001004226.1	kidney predominant protein Ncu-g1	2q34	1.54	0.0065
RGD1560263	XM_574989.2	similar to nuclear receptor binding factor 2 (Nrf2)	2q34	1.53	0.0045
Genes up-regulated in DA.ACI(Cia10)				**Cia10/DA**	
Gstm2	NM_177426.1	Glutathione S-transferase, mu 2	2q34	2.71	0.0028
Gstm1	NM_017014.1	Glutathione S-transferase, mu 1	2q34	2.58	0.0029
Nes	NM_012987.1	Nestin	2q34	2.06	0.0060
Tspan2	NM_022589.1	Tetraspanin 2	2q34	2.02	0.0057
Adamtsl4	NM_001034012.1	ADAM metallopeptidase with thrombospondin type 1 motif like	2q34	1.76	0.0077
Palmd	NM_001025688.1	Palmdelphin	2q41	1.64	0.0038
Pde5a	NM_133584.1	Phosphodiesterase 5A	2q42	1.58	0.0020