Research article

Cia5d regulates a new fibroblast-like synoviocyte invasion-associated gene expression signature

Teresina Laragione1, Max Brenner1, Wentian Li2 and Pércio S Gulko1,3

1Laboratory of Experimental Rheumatology, Center for Genomics and Human Genetics, Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, New York 11030, USA
2Genomics and Human Genetics, Feinstein Institute for Medical Research, 350 Community Drive Manhasset, New York 11030, USA
3Department of Medicine, New York University School of Medicine, 550 First Avenue, New York, 10016, USA

Corresponding author: Pércio S Gulko, pgulko@nshs.edu

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Abstract

Introduction The in vitro invasive properties of rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLSs) have been shown to correlate with disease severity and radiographic damage. We recently determined that FLSs obtained from pristane-induced arthritis (PIA)-susceptible DA rats are also highly invasive in the same in vitro assay through Matrigel. The transfer of alleles derived from the arthritis-resistant F344 strain at the arthritis severity locus Cia5d (RNO10), as in DA.F344(Cia5d) congenics, was enough to significantly and specifically reduce the invasive properties of FLSs. This genetically controlled difference in FLS invasion involves increased production of soluble membrane-type 1 matrix metalloproteinase (MMP) by DA, and is dependent on increased activation of MMP-2. In the present study we aimed to characterize the pattern of gene expression that correlates with differences in invasion in order to identify pathways regulated by the Cia5d locus.

Methods Synovial tissues were collected from DA and DA.F344(Cia5d) rats 21 days after the induction of PIA. Tissues were digested and FLSs isolated. After a minimum of four passages, FLSs were plated on Matrigel-covered dishes at similar densities, followed by RNA extraction. Illumina RatRef-12 expression BeadChip arrays were used. Expression data were normalized, followed by t-test, logistic regression, and cluster analysis. Real-time PCR was used to validate the microarray data.

Results Out of the 22,523 RefSeq gene probes present in the array, 7,665 genes were expressed by the FLSs. The expression of 66 genes was significantly different between the DA and DA.F344(Cia5d) FLSs (P < 0.01). Nineteen of the 66 differentially expressed genes (28.7%) are involved in the regulation of cell cycle progression or cancer-associated phenotypes, such as invasion and contact inhibition. These included Cxcl10, Vil2 and Nras, three genes that are upregulated in DA and known to regulate MMP-2 expression and activation. Nine of the 66 genes (13.6%) are involved in the regulation of estrogen receptor signaling or transcription. Five candidate genes located within the Cia5d interval were also differentially expressed.

Conclusions We have identified a novel FLS invasion associated gene expression signature that is regulated by Cia5d. Many of the genes found to be differentially expressed were previously implicated in cancer cell phenotypes, including invasion. This suggests a parallel in the behavior of arthritis FLSs and cancer cells, and identifies novel pathways and genes for therapeutic intervention and prognostication.

Introduction Rheumatoid arthritis (RA) is a common chronic autoimmune disease that affects approximately 1% of the population [1]. It is a complex trait, in which genetic and environmental factors mediate disease susceptibility and severity [1]. Basic joint pathology in RA is characterized by pronounced synovial hyperplasia, also called ‘pannus’, which produces several proinflammatory cytokines and proteases and, like a malignant tumor, invades and destroys cartilage and bone [2-4].

CXCR: C-X-C chemokine receptor; DMEM: Dulbecco’s modified Eagle’s medium; ER: estrogen receptor; FLS: fibroblast-like synoviocyte; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MMP: matrix metalloproteinase; MT1: membrane-type 1; PCR: polymerase chain reaction; RA: rheumatoid arthritis.
The formation of the synovial pannus is regulated by complex interactions between synovial resident cells and infiltrating inflammatory cells [5,6], and their production of paracrine and autocrine factors such as cytokines and growth factors [7-9], nuclear factor-kB activation [10], and angiogenesis [11]. The fibroblast-like synoviocyte (FLS) is a key player in this process, and its numbers are markedly increased in the hyperplastic synovial pannus of RA and rodent models of arthritis [4]. RA FLSs invade cartilage [12] and produce increased amounts of several proteolytic enzymes that further contribute to joint destruction [2,3]. The invasive properties of RA FLSs have also been associated with radiographic damage in RA, a parameter of disease severity, which emphasizes their direct clinical relevance [13].

We have previously identified Cia5d as an arthritis severity locus and showed that DA.F344(Cia5d) rats congenic for this interval developed significantly milder arthritis, with nearly no pannus formation and neither bone nor cartilage destruction, as compared with highly susceptible DA rats [14]. We also determined that Cia5d regulates the invasive properties of FLSs, thus providing an explanation for its role in joint damage [15]. The arthritis gene located within Cia5d controls the FLS production of soluble membrane-type 1 (MT1)-matrix metalloproteinase (MMP) and activation of MMP-2 [15]. This was the first time that FLS phenotypes were found to be genetically regulated.

In the present study we took advantage of this genetically regulated FLS invasive phenotype and compared highly invasive with minimally invasive cells’ gene expression signatures using microarrays. The study of more than 22,000 genes identified a gene expression signature related to invasion that is differentially regulated between FLSs from DA and DA.F344(Cia5d) rats. The novel FLS invasion pathways described here resemble those described in cancer cell lines and have the potential to become novel targets for therapeutic intervention.

Materials and methods

Rats

DA (DA/BklArbNsi, arthritis-susceptible) inbred rats (originally from Bentin & Kingman, CA, USA) were maintained at the Arthritis and Rheumatism Branch (Arb; National Institutes of Health) and then transferred to the Feinstein Institute (previously named North Shore-LIJ Institute; Nsi). The genotype-guided breeding of DA.F344(Cia5d) was previously described in detail [14]. Briefly, a 37.2 megabase interval on rat chromosome 10 was transferred from F344 into the DA background over 10 backcrosses followed by at least five intercrosses (Figure 1). The experiments were conducted with rats homozygous at the congenic interval. All experiments involving animals were reviewed and approved by the Feinstein Institute for Medical Research Institutional Animal Care and Use Committee. Animals were housed in a pathogen free environment, on 12-hour light and dark cycles, with free access to food and water.

Induction of PIA and arthritis scoring

Rats aged 8 to 12 weeks received 150 μl of pristane by intradermal injection divided into two sites at the base of the tail [14,16]. The animals were scored on days 14, 18 and 21 after pristane induction using a previously described arthritis scoring system [17,18]. On day 21 after injection, the animals were killed and synovial tissue was collected from the ankles for FLS isolation.

Isolation and culture of primary FLS

FLSs were isolated by enzymatic digestion of the synovial tissue. Briefly, tissues were minced and incubated with a solution containing DNase 0.15 mg/ml, hyaluronidase type I-S 0.15 mg/ml, and collagenase type IA 1 mg/ml (Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Invitrogen Corporation, Carlsbad, CA, USA) for 1 hour at 37°C. Cells were washed and re-suspended in DMEM supplemented with 10% fetal bovine serum (Gibco), glutamine 30 mg/ml, amphotericin B 250 μg/ml (Sigma), and gentamicin 10 mg/ml (Gibco). After overnight culture, nonadherent cells were removed and adherent cells were cultured. All experiments were performed with cells after passage four (95% FLS purity).

Flow-cytometric characterization of FLSs

Freshly trypsinized FLSs (105) were re-suspended in phosphate-buffered saline with 0.02% azide (Sigma-Aldrich) and...
1% bovine serum albumin (P Biomedicals, Aurora, OH, USA), and incubated with 1 μg anti-CD32 (Pharmingen, San Diego, CA, USA) to block Fcγ II receptors. Cells were stained with saturating concentrations of CD90 (OX-7; PerCP, Pharmin- gen) or isotype control. Stained cells were fixed with 1% paraformaldehyde in phosphate-buffered saline and analyzed by flow cytometry in a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA), using the BD Cell-Quest™ Pro version 4.0.1 software (Becton Dickinson).

**FLS culture on Matrigel**

We previously studied the invasive properties of FLSs through a collagen matrix (Matrigel). Cell interactions with the extracellular matrix are known to influence the expression of several genes, including activation of MMP-2 [19], which is a key mediator of the FLS invasive phenotype. Therefore, in order to study the gene expression signature of highly invasive and minimally invasive FLSs, cells were cultured under the same conditions as used in the invasion studies. Specifically, 100% confluent 75 cm² FLS culture flasks were trypsinized (trypsin 0.25% with EDTA 0.1%). The rates of cellular proliferation differed among cell lines, and we previously showed that FLS proliferation does not correlate with the FLS invasive behavior. In order to have similar cell confluence at the time of FLS harvesting for RNA extraction, 10% to 50% of the high-density 75 cm² cell culture flasks (depending on the cell line) were plated in Matrigel-coated 10 cm culture dishes (Becton Dickinson) with DMEM, 10% fetal bovine serum, antibiotics, and glutamine. Cell cultures were maintained at 37°C with 5% carbon dioxide for 24 hours. After 24 hours, FLSs were harvested using a cell scraper (Corning, Acton, MA, USA) followed by digestion of the Matrigel with 10 ml collagenase D 1 mg/ml (Roche Applied Science, Indianapolis, IN, USA) at 37°C for 10 minutes. FLSs were then collected by centrifugation, washed twice with ice-cold phosphate-buffered saline. Cell pellets were re-suspended in RLT lysis buffer (RNeasy Mini Kit; Qiagen, Valencia, CA, USA) with 1% (vol/vol) β-mercaptoethanol (Sigma). Cell-lysis buffer suspension was vortexed, frozen in liquid nitrogen and stored at -80°C until RNA extraction.

**RNA extraction and quality assessment**

Cells in RLT buffer were disrupted using QIAshredder spin columns (Qiagen), and total RNA was extracted using the RNeasy Mini Kit (Qiagen), in accordance with the manufacturer’s instructions. Samples were digested with DNase (Qiagen) and eluted with 30 μl RNase-free water. RNAs were quantified and assessed for purity using a NanoDrop spectrophotometer (Rockland, DE, USA). RNA integrity was verified with a BioAnalyzer 2100 (Agilent, Palo Alto, CA, USA).

**RNA preparation and microarray experiments**

The RatRef-12 Expression BeadChip contains 22,524 probes for a total of 22,228 rat genes selected primarily from the NCBI RefSeq database (Release 16; Illumina, San Diego, CA, USA), and was used in accordance with the manufacturer's instructions. All reagents have been optimized for use with Illumina’s Whole-Genome Expression platform. Total RNA 200 ng was used for cRNA in vitro transcription and labeling with the TotalPrep™ RNA Labeling Kit using Biotinylated-UTP (Ambion, Austin, TX, USA). Hybridization is carried out in Illumina Intellihyb chambers at 58°C for 18.5 hours, which is followed by washing and staining, in accordance with the Illumina Hybridization System Manual. The signal was developed by staining with Cy3-streptavidin. The BeadChip was scanned on a high resolution Illumina BeadArray reader, using a two-channel, 0.8 μm resolution confocal laser scanner.

**Data extraction and normalization**

The Illumina BeadStudio software (Version 2.0) was used to extract and normalize the expression data (fluorescence intensities) for the mean intensity of all 12 arrays. Genes expressed in all 12 arrays were selected for analyses. Normalized data were analyzed using the t-test and logistic regression.

**Statistics and analyses**

The t-test was used to compare means of the log-transformed and non-log-transformed data. Genes with a P value under 0.01 between DA and DAF344(Cia5d) were considered significant and included in additional analysis. The logistic regression model fitting was carried out as previously described [20,21] using the filtered gene list. The statistical significance of a logistic regression result was obtained by comparing the deviance with the 'null deviance'. This null deviance is the (-2)log-likelihood of a random model in which the probability for a sample to belong to a group (for example, DA) is equal to the proportion of DA samples in the dataset. The difference between the deviance and the null deviance follows the χ² distribution with one degree of freedom by chance alone, and this χ² distribution was used to determine the P value. The R statistical package [22] was used for t-test and logistic regression analyses.

The Ingenuity IPA 5.5.1 program (Ingenuity, Redwood City, CA, USA) and PubMed and GEO (Gene Expression Omnibus) searches were used for pathways detection. CLUSTER [23] and TREEVIEW [24] were used for cluster analysis and generation of a heat map.

**Quantitative real-time PCR**

The same RNA used for the microarray experiments was also used for the quantitative real-time PCR confirmation experiments. Total RNA 200 ng from each sample was used for cDNA synthesis using the Superscript III kit (Invitrogen). Primers and probe sequences were designed to target the same exon as used in the Illumina RatRef-12 Expression BeadChip. We used Exiqon (Woburn, MA, USA) and Taqman (ABI, Applied Biosystems, Foster City, CA) probes (Table 1). GAPDH was used as endogenous control. Probes were labeled with FAM at the 5’ end and TAMRA at 3’ end and used at a final concentration of 100 nmol/l. Primers were used at
200 nmol/l concentration with Eurogentec quantitative real-time PCR mastermix (Eurogentec, San Diego, CA, USA). The ABI 7700 quantitative real-time PCR thermocycler was used at 48°C for 30 minutes, 95°C for 10 minutes, and 45 cycles of 95°C for 0.15 minutes and 60°C for 1 minute. Samples were run in duplicates and the mean s used for analysis. Data were analyzed using Sequence Detection System software version 1.9.1 (ABI). Results were obtained as Ct (threshold cycle) values. Relative expression of all the genes was adjusted for GAPDH in each sample (ΔCt), and ΔCt used for t-test analysis. Quantitative real-time PCR fold differences were calculated with 2-ΔΔCt [25].

### Results

#### Characterization of the FLS cell lines used

In previous studies we determined that DA FLSs were highly invasive, and that alleles derived from the arthritis-resistant strain F344 at the Cia5d interval, as in DA.F344(Cia5d) congenics (Figure 1), specifically reduced the invasive properties of FLSs. Additionally, FLSs from DA and DA.F344(Cia5d) strains expressed similar mRNA levels of transforming growth factor-β, tumor necrosis factor-α, IL-1β and IL-6, as well as MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, MT1-MMP and MT2-MMP [15]. Both strains had similar collagenase and MMP-3 activity, but levels of soluble MT1-MMP and active MMP-2 were increased in DA. MMP-2 inhibition reduced DA FLS invasion to levels similar to those of DA.F344(Cia5d). Cytoskeleton characteristics were also similar in DA and DA.F344(Cia5d) FLSs [15].

In the present study FLSs were stained with CD90, a marker for FLS [26], and analyzed by flow cytometry. Comparable numbers of CD90+ cells were detected both in five different DA and five different DA.F344(Cia5d) rats (percentage of CD90+ cells [mean ± standard deviation]: DA 95.46 ± 8.9 and DA.F344 [Cia5d] 96.51 ± 5.9), demonstrating that the cell lines were homogeneously CD90+.

#### Genes expressed by FLSs and filtering criteria

A total of 7,665 genes out of 22,228 genes represented in the Illumina RatRef-12 BeadChip were expressed by both DA and DA.F344(Cia5d) FLSs. Log transformation did not significantly affect the list of differentially expressed genes, and therefore results are shown from analyses done with non-log-transformed data.

### Table 1

| Genes studied with QPCR for confirmatory studies, primers and probe sequences |
|---|
| Accession number | Gene symbol | Target exon | Probe | Forward primer | Reverse primer |
|---|---|---|---|---|---|
| **Up-regulated in DA** | | | | | |
| NM_1390089.1 | Cxcl10 | 4 | Exiqon Universal probe 67 | TTCGGACCAGCTCTTAGAGAA | GCCGTGCTCACAGAAAGAG |
| XM_225552.3 | Trim16 | 6 | Exiqon Universal probe 1 | GTGAACCTCTCCCACTCCA | CAGCTGATTTTGGGACAG |
| NM_017207.1 | Trpv2 | 15 | Exiqon Universal probe 6 | CTTCTCACTCTTTCCAGAGGA | GACCTGAAGGGGCGAG |
| NM_019357.1 | Vif | 13 | CCCAAAGACCCGTGGAA TCCCTCA | GGTACCGGGGCATGTCT | GGCGCTGTGGGCACTATG |
| LOC309362 | Dnmrp | 16 | Exiqon Universal probe 97 | TTGCTCTAGCATGGTCCTTA | ACCAGGATTTTAGGGGAC |
| NM_001107408 | Gins3 | 3–4 | Exiqon Universal probe 17 | GTCGACGCACCCCAACAAAT | GAAACGGTCAATTAAAGTCT |
| **Down-regulated in DA** | | | | | |
| XM_235434.4 | Gsdmdc1 | 13 | Exiqon Universal probe 68 | AGCAGGCTTGAGAAACAGACG | TCCCTACCCACAGCTCC |
| XM_222868.4 | Olfl12b | 8 | Exiqon Universal probe 106 | CTCCTCTTCTCATGTCTCT | GCAAGGCCCAAGGAAAT |
| NM_001038321.1 | Gadd45b | 4 | Exiqon Universal probe 25 | ACAGGGTGTCGCAAAGAC | CCAGGCCATGCTCATAATG |
| **Estrogen receptors** | | | | | |
| NM_012868.1 | Esr1 | - | Exiqon Universal probe 67 | GCAAGAATGTCGCTGCTTCT | TGAAGACGTGAGCATCCAG |
| NM_012754 | Esr2 | - | Exiqon Universal probe 94 | CTTGAGAGCGTTCTCGGTGTA | CAGAACCCTTCAGATGGT|

**aTaqman probe.** **bSame region used in the Illumina microarray.**
Table 2

| Gene Symbol | Definition | Accession number | DA mean | Cia5d mean | Fold change | P value | Overall rank |
|-------------|------------|------------------|---------|------------|-------------|---------|--------------|
| **Cancer, Cell Cycle, DNA replication, recombination and repair** | | | | | | | |
| Trim16_predicted | Tripartite motif protein 16 (predicted) | XM_220552.3 | 262.14 | 82.27 | -3.2 | 0.0033 | 23 |
| Cxcl10 | Chemokine (C-X-C motif) ligand 10 | N1M_139089.1 | 1218.54 | 434.48 | -2.8 | 0.0001 | 2 |
| Dnmbp | Similar to Dynamin binding protein (Scaffold protein Tuba) | XM_219860.3 | 739.97 | 385.61 | -1.9 | 0.0088 | 62 |
| **W2** | Villin 2 (Ezrin) | N1M_019357.1 | 1642.95 | 984.09 | -1.7 | 0.0023 | 15 |
| Nras | Neuroblastoma RAS viral (v-ras) oncogene homolog | XM_579607.1 | 910.25 | 601.06 | -1.5 | 0.0087 | 60 |
| Brms1l_predicted | Breast cancer metastasis-suppressor 1-like (predicted) | XM_216712.3 | 187.93 | 125.37 | -1.5 | 0.0094 | 64 |
| Hnrd | Heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37 kDa) | XM_024404.1 | 2909.16 | 1959.49 | -1.5 | 0.0010 | 8 |
| Rpa2 | Replication protein A2 | N1M_021582.1 | 1583.81 | 1154.73 | -1.4 | 0.0074 | 48 |
| Ube2d3 | Ubiquitin-conjugating enzyme E2D 3 | N1M_031237.1 | 123.48 | 99.45 | -1.2 | 0.0017 | 10 |
| Lsn8_predicted | LSM8 homolog, U6 small nuclear RNA associated (S. cerevisiae) (predicted) | XM_216102.3 | 3766.75 | 3121.49 | -1.2 | 0.0024 | 16 |
| Smc1l1 | Structural maintenance of chromosomes 1 like 1 (S. cerevisiae) | N1M_031683.1 | 4684.45 | 3923.73 | -1.2 | 0.0044 | 30 |
| Rpa3_predicted | Replication protein A3 (predicted) | XM_216097.3 | 4013.83 | 3410.52 | -1.2 | 0.0022 | 14 |
| **Cell Signaling** | | | | | | | |
| Stip1 | Stress-induced phosphoprotein 1 (Stip1) | N1M_138911.2 | 3478.09 | 2568.75 | -1.4 | 0.0028 | 18 |
| **Ubiquitination** | | | | | | | |
| Usp24_predicted | Ubiquitin specific protease 24 (predicted) | XM_233260.3 | 111.07 | 74.14 | -1.5 | 0.0037 | 25 |
| Stub1_predicted | STIP1 homology and U-Box containing protein 1 (predicted) | XM_213270.3 | 4967.20 | 4164.69 | -1.2 | 0.0034 | 24 |
| **Ribosomal Proteins** | | | | | | | |
| Rps6 | Ribosomal protein S6 (Rps6) | N1M_017160.1 | 29306.46 | 24538.18 | -1.2 | 0.0085 | 57 |
| LOC300278 | Similar to 40S ribosomal protein S9 | XM_213106.3 | 28115.69 | 26209.24 | -1.1 | 0.0086 | 59 |
| LOC367102 | Similar to 40S ribosomal protein S9 | XM_345948.2 | 25678.47 | 23353.32 | -1.1 | 0.0043 | 28 |
| **Others** | | | | | | | |
| Trpv2 | Transient receptor potential cation channel, subfamily V, member 2 | N1M_017207.1 | 177.90 | 92.25 | -1.9 | 0.0075 | 49 |
| Gins3_predicted | GINS complex subunit 3 (Psf3 homolog) | XM_226235.2 | 171.57 | 89.64 | -1.9 | 0.0010 | 6 |
| LOC499310 | Similar to cell division cycle associated 5 | XM_574612.1 | 450.69 | 270.81 | -1.7 | 0.0061 | 44 |
| LOC298186 | Similar to hypothetical protein FLJ33868 (predicted) | XM_238399.3 | 271.10 | 177.29 | -1.5 | 0.0070 | 46 |
| Terf1_predicted | Telomeric repeat binding factor 1 (predicted) | XM_238387.3 | 98.95 | 66.02 | -1.5 | 0.0048 | 34 |
| LOC308004 | Similar to hypothetical protein FLJ13188 (predicted) | XM_217663.3 | 573.01 | 383.19 | -1.5 | 0.0083 | 56 |
| LOC310177 | Similar to RIKEN cDNA 0610040D20 | XM_225872.2 | 85.32 | 58.03 | -1.5 | 0.0044 | 29 |
thermore, cluster analysis separated DA FLSs from DA.F344(Cia5d) FLSs, demonstrating that the two strains could be reliably differentiated by gene expression (Figure 2).

**Genes upregulated in the highly invasive DA FLSs and downregulated in DA.F344(Cia5d) include cancer-associated and invasion regulatory genes**

Cluster analysis identified three main clusters among the genes expressed in increased levels in DA (Figure 2). One of the three clusters contained eight genes, three of which have been implicated in cancer and cancer-related cellular phenotypes such as invasion, and included Cxcl10, Vil2 and Dnmbp (Figure 3). The other genes in this cluster are involved in ion transport (Trpv2), mitosis (Smc1L1), or have incompletely characterized functions (Trim16, Ranbp6 and Hnpp1L2). In total, 12 out of the 36 genes (33.3%) expressed in increased levels by DA FLSs and downregulated in DA.F344(Cia5d) are known to regulate cancer-associated processes, including cell cycle progression (Rpa2 and Rpa3), cell invasion (Cxcl10, Vil2, Nras, and Dnmbp), and metastasis (Vil2 and Brms1l), respectively (Table 2). In fact, Cxcl10 was the second best discriminator between DA and DA.F344(Cia5d) cell lines, as per logistic regression (Table 2).

Of additional interest in relation to the MMP-2-dependent difference in FLS invasion that we have observed, three of these genes – namely Cxcl10, Vil2 and Nras – are known to regulate the synthesis or activation of gelatinases. Increased levels of Cxcl10, Vil2, Dnmbp, Trim16, and Trpv2 in DA were confirmed using quantitative real-time PCR, with most of these genes having a nearly fourfold or greater difference in expression (P < 0.05; Figure 4a).

**Genes downregulated in the highly invasive DA FLSs and upregulated in DA.F344(Cia5d) include tumor suppressor and cell cycle check-point genes**

The list of genes with reduced expression in DA, as compared with increased expression in DA.F344(Cia5d) congenics, included seven genes that are involved in tumor suppression-like activity and cell cycle check-points, such as Aph1a, Brwd3, Gadd45b, Gmfg, Lox, and Plekhg2 (Table 3). Gadd45b was chosen for quantitative real-time PCR confirmation (P < 0.05; Figure 4b). These observations, combined with the 11 cancer and invasion associated genes upregulated in DA, suggest an invasion-favoring profile similar to that described in cancer cells, characterized by reduced expression cell cycle check-point and tumor suppressor genes combined with increased expression of invasion genes.

**Additional genes with reduced expression in DA FLSs**

Additionally, Ubxd2, Fzd4, Fkbp7, Olfml2b, Gsdmdc1 and the transcriptional co-repressor Ncor1 were among the genes downregulated in DA and with increased expression in DA.F344(Cia5d). Gtfl3b (predicted), a gene trap fragment with unknown function, was among the most significantly differentially expressed genes (P = 0.000025; 2.2-fold difference; Table 3). The greater than twofold difference in expression of Olfml2b and Gsdmdc1 was confirmed with quantitative real-time PCR (Figure 4b).

**Increased number of estrogen-inducible and ER signaling regulatory genes among the differentially expressed genes**

Nine genes or 13.6% of the 66 differentially expressed genes were either estrogen-inducible genes, such as Cxcl10, Vil2,
Table 3

Genes with increased expression in synovial fibroblasts from DA.F344 (Cia5d) compared with DA

| Gene Symbol       | Definition                                                                 | Accession number | DA mean  | Cia5d mean | Fold change | P value | Overall rank |
|-------------------|----------------------------------------------------------------------------|------------------|----------|------------|-------------|---------|--------------|
| **Cancer, Cell Cycle, DNA replication, recombination and repair** |
| Gadd45b           | Growth arrest and DNA-damage-inducible 45 beta                            | NM_001008321.1   | 214.12   | 412.97     | 1.9         | 0.00572 | 39           |
| Gmfg              | Glia maturation factor, gamma (Gmfg)                                      | NM_181091.2      | 1359.39  | 2261.87    | 1.7         | 0.00817 | 54           |
| Plekg2_predicted  | Pleckstrin homology domain containing, family G (with RhoGef domain) member 2 (predicted) | XM_214862.3      | 91.97    | 147.62     | 1.6         | 0.00784 | 52           |
| Lox               | Lysyl oxidase                                                              | XM_579391.1      | 15755.11 | 24559.79   | 1.6         | 0.00198 | 12           |
| Brwd3_predicted   | Similar to bromo domain-containing protein disrupted in leukemia (LOC317213) | XM_228518.3      | 43.85    | 52.99      | 1.2         | 0.00596 | 43           |
| Aph1a             | Similar to anterior pharynx defective 1 homolog A (C. elegans)             | XM_345251.2      | 2820.66  | 3246.28    | 1.2         | 0.00046 | 4            |
| Pex19_predicted   | Peroxisome biogenesis factor 19 (predicted)                               | XM_225711.3      | 119.41   | 135.98     | 1.1         | 0.00561 | 38           |
| **Cell Signaling** |
| Fkbp7_predicted   | FK506 binding protein 7 (predicted)                                       | XM_215758.3      | 784.02   | 1450.53    | 1.9         | 0.00578 | 40           |
| Ncor1             | Nuclear receptor co-repressor 1                                            | XM_577103.1      | 420.35   | 679.65     | 1.6         | 0.00454 | 32           |
| Tap1              | Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)               | NM_032055.1      | 190.32   | 288.49     | 1.5         | 0.00878 | 61           |
| Prnp              | Prion protein                                                              | XM_579340.1      | 17242.89 | 24050.29   | 1.4         | 0.00029 | 3            |
| Fzd4              | Frizzled homolog 4 (Drosophila)                                           | NM_022623.1      | 44.45    | 60.14      | 1.4         | 0.00406 | 26           |
| **Gene expression** |
| H1f0              | H1 histone family, member 0                                                | NM_012578.2      | 150.12   | 229.40     | 1.5         | 0.00707 | 47           |
| **Cell-Cell Interaction**|
| Fath              | Hypothetical gene supported by NM_031819; Fath fat tumor suppressor homolog (Drosophila) | XM_579538.1      | 3803.04  | 5806.86    | 1.5         | 0.00206 | 13           |
| **Extracellular Matrix** |
| Col5a1            | Collagen, type V, alpha 1 (Col5a1)                                         | NM_134452.1      | 7240.26  | 9852.22    | 1.4         | 0.00807 | 53           |
| **Others**        |
| Gtlf3b_predicted  | Gene trap locus F3b (predicted)                                            | XM_343907.2      | 78.16    | 175.41     | 2.2         | 0.00003 | 1            |
| Olfm2b_predicted  | Olfactomedin-like 2B (predicted)                                          | XM_222868.3      | 1336.30  | 2949.43    | 2.2         | 0.00241 | 17           |
Trim16, Gins3 (predicted), and Gadd45b, or genes involved in modulating the estrogen receptor (ER) signaling such as Stub1 and Stip1. Ncor1 negatively regulates ER-mediated transcription and its levels were also reduced in DA, further suggesting unopposed ER-mediated transcription. The differential expression of Cxcl10, Vil2, Trim16, Gins3, and Gadd45b was confirmed with quantitative real-time PCR (Figure 4a, b). The ERs Esr1 and Esr2 were not differentially expressed in the microarray analysis, and those results were confirmed with quantitative real-time PCR (Figure 4b). There was a trend toward increased expression Esr2 in DA.F344(Cia5d), but that difference did not reach statistical significance \( (P = 0.093; \) Figure 4b). Taken together, this pattern of gene expression suggests that the invasive DA FLSs have an enhanced ER activity regulated at different levels that could include reduced degradation of the ER, reduced inhibition of the ER-mediated transcription, and increased levels of estrogen-inducible genes.

Five of the differentially expressed genes are located within the Cia5d interval

Five out of the 66 differentially expressed genes were located within the Cia5d interval (Table 4). The number of genes located within the Cia5d interval found to be differentially expressed between DA and DA.F344(Cia5d) FLSs was greater than would be expected by chance (3.3% observed versus 0.8% expected by chance; \( P = 0.0044 \) by \( \chi^2 \) with Yates correction; Table 5).

Discussion

RA histology is typically characterized by pronounced synovial hyperplasia, also called ‘pannus’. The RA pannus produces proinflammatory cytokines and proteases, and invades cartilage and bone leading to joint destruction and deformities [4]. The FLS is a key player in RA pannus and joint pathology, and has increased invasive properties, compared with osteoarthritis, even after several passages in vitro [12,27]. Furthermore, the increased invasive properties of RA FLSs have been associated with increased radiographic joint destruction [13], underscoring the relevance of this in vitro phenotype to disease outcome.

We recently described the first evidence that the invasive properties of FLSs are genetically regulated [15]. We determined that a gene located within the arthritis severity regulatory Cia5d interval specifically controls the invasive properties of FLSs via the regulation of the production of soluble MT1-MMP and activation of MMP-2 [15]. Levels of active MMP-2 are also increased in the synovial fluid of patients with RA, and correlate with disease severity and radiographic damage [28]. Therefore, understanding the regulation of cell invasion and

### Table 3 (Continued)

| Genes with increased expression in synovial fibroblasts from DA.F344 (Cia5d) compared with DA |
|-----------------------------------------------|
| Gsdmdc1_predicted | Gasdermin domain containing 1 (predicted) | XM_235434.3 | 458.74 | 831.39 | 1.8 | 0.00295 | 20 |
| Trim41_predicted | Tripartite motif-containing 41 (predicted) | XM_220357.3 | 422.66 | 732.37 | 1.7 | 0.00100 | 7 |
| LOC498B15 | Hypothetical gene supported by AY771707 | XM_579873.1 | 245.56 | 366.68 | 1.5 | 0.00281 | 19 |
| LOC30460 | Similar to N-acetylneuraminic pyruvate lyase | XM_222736.3 | 270.64 | 401.65 | 1.5 | 0.00176 | 11 |
| Setdb2_predicted | SET domain, bifurcated 2 (predicted) | XM_224248.3 | 94.38 | 136.31 | 1.4 | 0.00945 | 66 |
| LOC361448 | Similar to cDNA sequence BC013529 (predicted) | XM_341726.2 | 2852.12 | 4043.46 | 1.4 | 0.00071 | 5 |
| LOC360899 | Similar to SERTA domain containing 4 | XM_341174.2 | 1771.29 | 2489.20 | 1.4 | 0.00886 | 63 |
| Ormd2_predicted | ORM1-like 2 (S. cerevisiae) (predicted) | XM_213832.3 | 1996.56 | 2773.15 | 1.4 | 0.00549 | 37 |
| LOC498067 | Similar to RIKEN cDNA 2310003P10 (LOC498067), mRNA | XM_573266.1 | 368.00 | 494.10 | 1.3 | 0.00860 | 58 |
| Nit1 | Nitrilase 1 | NM_182668.1 | 3397.58 | 4472.84 | 1.3 | 0.00296 | 21 |
| Fam18b_predicted | Family with sequence similarity 18, member B (predicted) | XM_219680.3 | 2915.92 | 3746.20 | 1.3 | 0.00447 | 31 |
| Ubxd2_predicted | UBX domain containing 2 (predicted) | XM_573443.1 | 2018.75 | 2569.23 | 1.3 | 0.00411 | 27 |

*Estrogen; ER, estrogen-induced, or estrogen-receptor signaling or degradation are marked in bold. *t* test. *Order (logistic regression) in the list of 66 genes differentially expressed between DA and DA.F344(Cia5d). *Cancer and invasion associated genes are in italics. *Increased expression in invading breast cancers.
MMP-2 activation is highly relevant to RA. In addition, several common cancers have increased levels of MMP-2, which correlates with worse prognosis [29-36], suggesting that identifying the Cia5d gene and the pathways controlled by it could potentially generate novel targets relevant to cancer treatment as well.

In the present study we used a novel strategy to identify differences in gene expression that correlate with the invasive properties of FLSs. First, two closely related strains were used. These strains have identical DA genomes, except that DA.F344(Cia5d) congenics have F344 arthritis-resistant alleles in a 37.2 megabase interval on chromosome 10. This strategy minimized noise related to allelic variations at other regions of the genome that are not related to the phenotype of interest. Second, instead of using synovial tissues, which have mixed cellularities that interfere with the interpretation of the results, we generate and used primary FLS cell lines. Third, FLSs from DA and DA.F344(Cia5d) differ in their invasive properties, thus providing a more precise phenotype. Finally, the cells used for RNA extractions were cultured on the same collagen matrix (Matrigel) used in the invasion experiments, hence recreating the same in vitro environment. This latter aspect is critical because extracellular matrix and cell influence processes that are central to cell invasion, such as the expression of adhesion molecules and MMP-2 activation [19], and...
are required for proper activation of the invasive phenotype, including gene transcription. This strategy led to the identification of new genes involved in FLS invasion.

A genome-wide analysis of gene expression conducted with RA FLSs suggested two patterns that correlated with increased or reduced inflammation in the tissues of origin [37]. Those RA FLSs were not studied for invasion, and there was no control group without erosive changes for comparison. Furthermore, the RNA was obtained from cells cultured on plastic dishes and not on a collagen matrix such as Matrigel. Therefore, it was not surprising that using different methodologies to address a different question we detected a new FLS invasion signature that is different from the two RA FLS gene expression patterns previously reported.

A genome-wide microarray-based gene expression analysis was conducted to identify genes and pathways that are differentially expressed between highly invasive DA and minimally invasive DA.F344(Cia5d) FLSs. The analysis revealed that 66 genes out of the 7,665 genes expressed by FLSs were differentially expressed between DA and DA.F344(Cia5d) FLSs (P < 0.01). Nineteen of the 66 differentially expressed genes (28.7%) had previously been implicated in tumor suppression activity or other cancer cell phenotypes, but had not been implicated in the invasive properties of the FLSs. These cancer-related phenotypes include malignant transformation (Hnrpd) [38], tumor growth (Ach1a and Gfmg) [39,40], oncogene-like activity (Pleckg2) [41], tumor apoptosis (Gadd45b) [42], tumor suppressor activity (Brwd3) [43], cancer cell growth arrest (Ube2d3) [44], contact inhibition (Gmfg) [45], and cell invasion (Lox, Ach1a, Cxcl10, Vi12, and Nras) [46-50]. Genetic variations in DNA synthesis gene Rpa3 have been associated with susceptibility to carcinomas [51], whereas increased cancer expression of Rpa2 is associated with adverse outcome in colon cancer [52]. Some of these genes were found to be expressed in increased levels in certain cancers (Hnrpd and Lsm8) [53,54], including highly invasive types [55]. These observations suggest that FLSs derived from arthritis joints and cancer cells share common processes in the regulation of cell invasion, and that these processes are in part regulated by a gene located within the arthritis severity locus Cia5d.

Nras [56,57], Vi12 (encoding the ezrin protein) [49,50], and Cxcl10 [58] – three genes that are upregulated in DA but downregulated in DA.F344(Cia5d) – have also been implicated in the regulation of gelatinases’ expression and activation, including MMP-2 (Figure 5). These observations provide a direct link between the invasion and MMP-2 phenotypes that we have been studying and the gene expression signature regulated by the Cia5d locus. Furthermore, studies with RA synovial tissues [59,60] and RA FLSs [60] have also demonstrated increased expression of Cxcl10 both at mRNA and protein levels. Cxcl10 has also been shown to increase the production and activity of gelatinases in RA FLSs [61], underscoring the direct relevance of our in vitro discoveries to human disease.
In addition to the proinvasive and MMP-2 activating properties associated with Cxcl10 in FLSs, this chemokine can also attract C-X-C chemokine receptor (CXCR)3-expressing inflammatory cells such as memory T cells [62] and mast cells [59] into the joint, further contributing to disease severity. Indeed, recent studies that either targeted Cxcl10 [63] or its receptor CXCR3 [64] significantly ameliorated arthritis in rodents. Cxcl10, Vil2 [66], and Trim16 [67] – three of the most significantly upregulated genes in DA – are known to be induced by estrogens (Figure 5). A complete analysis of all of the 66 differentially expressed genes revealed that nine of them (13.6%) were either regulated by estrogen (Cxcl10, Vil2, Trim16, Gins3, Gadd45b, and Gmfg) [68] or are involved in ER signaling (Stip1), ER ubiquitination (Stub1), or ER-mediated transcription (Ncor1). These observations suggested that abnormalities in the regulation of ER signaling and ER-mediated transcription could contribute to the invasive properties of DA FLSs. Indeed, estrogens have been shown to increase levels of active MMP-2 in various tissues and cell types [69-71], including breast cancers [72], and estrogen antagonists reversed that effect [71,73]. Estrogens also increase the production of active MMP-2 and the in vitro invasive properties of RA FLSs [74] (Figure 5). Although estrogens are typically thought of as having anti-inflammatory properties [75], our observations suggest an intrinsic dysregulation in ER signaling in DA FLSs. This dysregulation in ER is controlled by the Cia5d gene, and could contribute to increased FLS invasion and cartilage and bone erosive changes.

Five of the differentially expressed genes were located within the Cia5d interval, and this number was greater than expected by chance. Three of these were upregulated in DA.F344(Cia5d) FLS (Ncor1, Trim41, and Gtlf3b) and two were downregulated in DA.F344(Cia5d) (Trpv2 and Trim16), raising the possibility that a polymorphism/mutation in one of these genes could explain the arthritis and FLS invasive phenotypes attributed to Cia5d. Specifically, a polymorphism in a regulatory element or intron in one of these genes, or in another gene in the region, could influence transcription, thus explaining differences in levels of mRNA and disease. This has been the case in studies of two other autoimmune or inflammatory diseases in which microarray analysis led to the identification of the disease-causing polymorphism [76,77]. In the present study only Ncor1, a transcriptional repressor regulated by estrogens, appears to be an interesting candidate. Trpv2 is a cation channel ubiquitously expressed, and the

### Table 4

**Differentially expressed genes located within the Cia5d interval on rat chromosome 10**

| Symbol | Definition | Accession number | Position (Mb) | Cytogenetic | DA mean | Cia5d mean | Fold change | t test | Overall rank |
|--------|------------|------------------|---------------|-------------|---------|------------|-------------|--------|--------------|
| **Reduced levels in Cia5d** | | | | | | | | | |
| Trim16 | Tripartite motif protein 16 (predicted) (Trim16_predicted) | XM_220552.3 | 48.95 | 10q23 | 262.14 | 82.27 | -3.19 | 0.00326 | 23 |
| Trpv2 | Transient receptor potential cation channel, subfamily V, member 2 (Trpv2) | NM_017207.1 | 48.76 | 10q23 | 177.90 | 92.25 | -1.93 | 0.00745 | 49 |
| **Increased levels in Cia5d** | | | | | | | | | |
| Ncor1 | Nuclear receptor co-repressor 1 (Ncor1) | XM_577103.1 | 48.62 | 10q23 | 420.35 | 679.65 | 1.62 | 0.00454 | 32 |
| Gtlf3b | Gene trap locus F3b (predicted) (Gtlf3b_predicted) | XM_343907.2 | 47.05 | 10q22 | 78.16 | 175.41 | 2.24 | 0.00003 | 1 |
| Trim41 | Tripartite motif-containing 41 (predicted) | XM_220357.3 | 34.08 | 10q21 | 422.65 | 732.37 | 1.73 | 0.00099 | 7 |

### Table 5

**A greater than expected number of genes located within the Cia5d interval were differentially expressed in FLS**

| Genes located within Cia5d | Differentially expressed | Not-differentially expressed |
|---------------------------|--------------------------|------------------------------|
| Genes located outside Cia5d | 61 (0.8%) | 7453 |

*p-value = 0.00442 (Chi-square with Yates correction).*
other three genes (Trim16, Trim41, and Gtlf3b) have less clear functions. The Cia5d interval contains more than 100 genes, and not all were present in the Illumina microarray. It would be premature to exclude these genes at this point, and additional studies with recombinant subcongenic strains are under way.

**Conclusion**

We have identified a novel invasion-associated gene expression signature and evidence suggesting a dysregulation in ER signaling in arthritis FLSs, which are regulated by the arthritis severity locus Cia5d. It is anticipated that the specific identification of the Cia5d gene, and the continued characterization of processes regulated by this gene, will generate new targets for therapeutic intervention aimed at reducing cartilage and bone destruction, and new prognostic markers for RA. The parallels between our findings in FLSs and observations from cancer studies suggest that the Cia5d gene might be important for cancer biology as well.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

All authors made substantial contributions to this study. TL generated the FLS cell lines and worked on the gene expression analyses. MB conducted the cellular and molecular biology experiments. WL worked on the gene expression statistical analysis. PSG designed the study and conducted the microarray gene expression analysis and pathway discovery, and wrote the manuscript. All authors read the manuscript critically, suggested modifications, and approved the final version.

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