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# Loss of imprinting of *IGF2* characterises high *IGF2* mRNA-expressing type of fibroblast-like synoviocytes in rheumatoid arthritis

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## ABSTRACT

**Objective** Increased expression of insulin-like growth factor 2 (*IGF2*) by fibroblast-like synoviocytes (FLS) was associated with low inflammatory synovium of patients with rheumatoid arthritis (RA). The aim of this study was to analyse whether the differential expression of *IGF2*, whose expression is normally restricted to one allele, is due to activation of the normally suppressed allele.

**Methods** *IGF2* gene expression of RA FLS was quantified by quantitative real-time PCR. FLS heterozygous for a 3'-untranslated region *IGF2* polymorphism were selected to measure the relative contribution of the allelic transcripts by allele-specific transcript quantification assay. Proliferation was determined by [<sup>3</sup>H]thymidine incorporation.

**Results** *IGF2* was shown to contribute to RA FLS proliferation. FLS could be classified in *IGF2* high and *IGF2* low-expressing cell lines. Allelic *IGF2* transcript quantification analysis revealed that in part of the RA FLS the normally suppressed allele was activated, resulting in biallelic expression of the *IGF2* gene. Biallelic expression was associated with increased levels of *IGF2* mRNA production.

**Conclusion** The findings indicate that the imprinting status of *IGF2* might underlie the increased expression of *IGF2*, which may contribute to autonomous growth of RA FLS of low inflammatory synovial tissues.

Rheumatoid arthritis (RA) is a disabling autoimmune disease characterised by chronic inflammation and hyperplasia of fibroblast-like synoviocytes (FLS) in the synovial membrane.<sup>1</sup> Rheumatoid FLS are involved in the maintenance and propagation of the inflammatory response and pannus formation, which invades and degrades the articular cartilage and bone. There is growing evidence to suggest that RA FLS can act as autonomous aggressors independent of immune cells.<sup>2</sup>

Gene expression profiling revealed that RA FLS derived from high-inflammatory (RA<sup>high</sup>) tissues were found to be associated with increased expression of an activin A/transforming growth factor beta (TGFβ)-induced gene programme, whereas FLS derived from low-inflammatory (RA<sup>low</sup>) tissues are associated with increased expression of insulin-like growth factor 2 (*IGF2*) and insulin-like growth factor binding protein (IGFBP5).<sup>3</sup> *IGF2* is an autocrine and paracrine growth factor involved in the embryonic and postnatal growth regulating cell survival, differentiation and proliferation.<sup>4</sup> Its biological effects are mainly mediated through its

interaction with insulin-like growth factor 1 receptor (IGF1R).<sup>5</sup> In most tissues, the transcription of *IGF2* is subject to so-called genomic imprinting, an epigenetic mechanism by which only one of the two alleles of a gene is expressed depending on its parental origin.

The maintenance of the imprinting status of *IGF2* is critical for the regulation of normal cell growth.<sup>4</sup> Deregulation of imprinted genes has been found in cancer.<sup>4,6</sup> In some cases, the 'silent' maternal allele was activated due to the loss of imprinting (LOI) resulting in biallelic expression of the original imprinted gene. In this study, we hypothesised that high expression of *IGF2* by a subset of FLS is due to the LOI of *IGF2*, and may contribute to the autonomous growth of these FLS.

## MATERIALS AND METHODS

### Culture of fibroblast-like synoviocytes

FLS (cultured to subconfluency (70%) in the fourth to seventh passage) were isolated from synovial tissue obtained perioperatively from osteoarthritis and RA patients, who fulfilled the American College of Rheumatology criteria for osteoarthritis<sup>7</sup> and RA,<sup>8</sup> respectively, and fracture patients. In this study, skin fibroblasts were also included.

### RA FLS proliferation assay

The proliferation of RA FLS (4×10<sup>5</sup> cells/well) was evaluated by measuring DNA synthesis. After 24 h culture in 0.5% fetal calf serum–Dulbecco's modified Eagles medium with 0.5% fetal calf serum and 1 μCi/well of [<sup>3</sup>H]thymidine FLS were lysed, transferred onto a membrane filter, and [<sup>3</sup>H]thymidine-incorporated DNA was quantified using a scintillation counter 1450 MicroBeta Plus (Wallac, Freiburg, Germany).

### Genotyping and allele-specific gene expression of *IGF2*

Genotyping was done by restriction fragment length polymorphism using the *ApaI/AvaII* polymorphism (rs680) present in the 3'-untranslated region (UTR) of exon 9 of the *IGF2* gene using a 236 bp genomic DNA PCR product (forward primer: 5'-CTTGGACTTTGAGTCAAATTGG-3'; and reverse primer: 5'-CCTCCTTTGGTCTTACTGGG-3'). The real-time (RT)-PCR products were digested with *ApaI* (G-allele)/*AvaII* (A-allele) (New England Biolabs, Frankfurt am Main, Germany). *ApaI* results in a 236 bp band fragment

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for the A allele and two fragments (173 bp and 63 bp) for the G allele. *Ava*II digestion results in a 214 bp fragment for the G allele and two bands of 151 bp and 63 bp for the A allele.

## Allele-specific transcript quantification

Allele-specific transcript quantification (ASTQ) was performed as previously described.<sup>9</sup> The 236 bp IGF2 reverse transcriptase-PCR fragment was labelled by final cycle labelling with [<sup>32</sup>P] cytosine to detect only de-novo synthesised PCR products, excluding heteroduplex PCR products due to mismatch annealing. The [<sup>32</sup>P]cytosine signal was corrected for the amount of cytosines present in the PCR fragment.

## Quantitative RT-PCR

The quantitative RT-PCR (qRT-PCR) was performed on an ABI PRISM 7900 HT System (Applied Biosystems, Foster City, California, USA). Gene expression levels were calculated relative to glyceraldehyde 3-phosphate dehydrogenase. Primer pairs are summarised in table 1.

## Statistical analysis

The statistical significance of the results was evaluated by unpaired Student's t test. Welch's correction was applied when the datasets under analysis had significantly different standard deviations. p Values of 0.05 or less were considered statistically significant (GraphPad Software, San Diego, California, USA).

## RESULTS

## IGF2 induces proliferation on RA FLS

To determine the effect of IGF2 on RA FLS proliferation, we measured DNA synthesis. After 24 h, FLS exposed to 50, 100 or 200 ng/ml IGF2 (which reflect the concentration present in the rheumatoid synovial fluid<sup>10</sup>) showed a 1.26, 2.00 or 2.36-fold increase in DNA synthesis compared with untreated cells, respectively. This response was neutralised with a blocking monoclonal antibody directed against IGF1R. This result indicates that exogenous IGF2 stimulates RA FLS proliferation in a dose-dependent manner through its interaction with IGF1R.

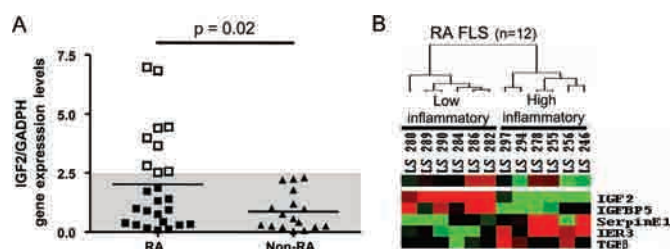
## Increased expression of IGF2 in a subset of RA FLS

To determine the IGF2 expression between RA and non-RA FLS, we performed a comparative analysis of IGF2 mRNA expression between RA FLS (n=25), osteoarthritis FLS (n=7), FLS from fractures (n=5) and skin fibroblast (n=5). This revealed that IGF2 mRNA expression was significantly higher in RA FLS (mean 2.01, SD 2.02) than non-RA controls (mean 0.87, SD 0.80; p=0.02; figure 1A).

Based on the differential IGF2 expression we could distinguish two subgroups of RA FLS. One subset of RA FLS (n=16) showed

similar IGF2 gene expression levels compared with non-RA controls and the other subset of RA FLS (n=9) showed high expression of mRNA IGF2 (figure 1A).

Previously, we found that increased IGF2 expression was accompanied by increased IGFBP5 expression and a low expression of genes that are involved in the TGFβ/activin A pathway. This pattern was characteristic of FLS derived from low inflammatory tissues.<sup>3</sup> In this study, we measured expression levels of IGF2, IGFBP5, TGFβ, immediate early response 3 (IER3) and SerpinE1, and showed that the 12 RA FLS tested clustered in two subgroups characterised by concomitant differential expression of IGF2 (p=0.04) and IGFBP5 (p=0.03) on one hand, and TGFβ (p<0.001) and the TGFβ/activin A-induced genes, IER3 (p=0.05) and SerpinE1 (p=0.08) on the other hand (figure 1B). These findings confirm previous observations of increased expression of IGF2 in a subgroup of RA FLS derived from low inflammatory tissues.



**Figure 1** Increased expression of insulin-like growth factor 2 (IGF2) mRNA in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS).

(A) This graph shows the gene expression levels of 25 RA FLS, and controls (non-RA) including osteoarthritis FLS (n=7), FLS from fractures (n=5) and skin fibroblast (n=5). Every spot represents a single sample and the lines represent the mean of the IGF2 expression of each group. We divided the RA group into IGF2 high or IGF2 low-expressing RA FLS using as a cut-off the mean + 1.96 × SD of the non-RA control group (0.87 + 1.57). The white-filled squares represent the IGF2 high-expressing FLS, whereas the black-filled squares represent the IGF2 low-expressing FLS. The grey area indicates the normal range of IGF2 expression within 95% confidence limits. The y-axis represents the levels of IGF2 mRNA gene expression relative to the endogenous reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as quantified by quantitative real-time (qRT)-PCR (unpaired Student's t test with Welch's correction, p=0.02). (B) The cluster diagram shows the unsupervised hierarchical clustering of the gene expression levels of IGF2, insulin-like growth factor binding protein 5 (IGFBP5), transforming growth factor beta (TGFβ), immediate early response 3 (IER3) and SerpinE1 in 12 RA FLS as measured by qRT-PCR. Gene expression levels were calculated relative to GAPDH. Each row represents a single gene; each column represents an individual FLS sample. Red colour indicates relative expression greater than the median of all samples, green colour indicates an expression lower than the median and black colour indicates equal expression levels. The clustering was performed using Cluster software (online at <http://rana.lbl.gov/EisenSoftware.htm>).

**Table 1** Primers used in the quantitative RT-PCR experiment

Gene	Size (bp)	Forward primer (5'→3')	Reverse primer (5'→3')
<i>IGF2</i>	134	CCTCCTGGAGACGTA CTGTGCT	TGGACTGCTCCAGGTGTCATA
<i>SerpinE1</i>	77	CCTGGGAATGACCGACATG	CGACGTGGAGAGGCTCTTG
<i>TGF</i>	135	TGACCTGGCCACCATTTCAT	TCCGTGGAGCTGAAGCAATAG
<i>IER3</i>	73	TGGGCGGACCATTAGGAAT	CTCGCAGCCACCCTAAAGG
<i>IGFBP5</i>	110	AGCTACCGCGAGCAAGTCA	TGTTTGGGCCGGAAGATCT
<i>GAPDH</i>	82	GCCAGCCGAGCCACATC	TGACCAGCGCCCAATAC

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IER3, immediate early response 3; IGF2, insulin-like growth factor 2; IGFBP5, insulin-like growth factor binding protein 5; RT, real-time; TGFβ, transforming growth factor beta.

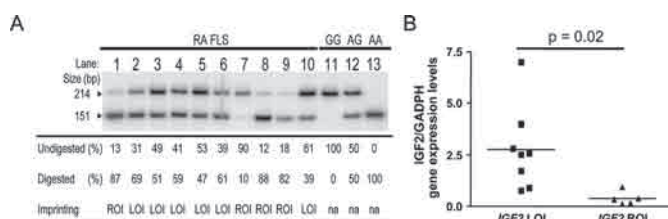
### Allele-specific gene expression of IGF2 in RA FLS

To analyse whether high IGF2 expression in RA FLS is associated with LOI of *IGF2* we investigated whether the subset of IGF2 high-expressing (IGF2<sup>high</sup>) FLS shows LOI. Therefore, we genotyped 25 RA FLS for the *Apal/AvalI* polymorphism located within the 3'-UTR of *IGF2* to select heterozygous RA FLS, which will allow us to determine the allelic origin of the IGF2 transcripts. We identified 13 out of 25 (52%) RA FLS that were heterozygous for this polymorphism.

We next determined whether IGF2 mRNA was expressed from a single allele or both alleles, that is, LOI. Therefore, we digested reverse transcriptase-PCR IGF2 products with *ApalI* and *AvalI* and quantified the relative expression of the respective alleles. As mRNA transcripts of the presumed 'silent' allele may have some residual expression, we established an arbitrary threshold to define the imprinting status.<sup>11</sup> According to these findings, we defined cases in which the presumed 'silent' allele represents less than 20% of the total IGF2 mRNA as reflecting significant silencing of the imprinted allele, that is, retention of imprinting (ROI). Using this threshold we observed that five out of 13 informative RA FLS showed ROI, whereas eight RA FLS showed LOI of *IGF2* (figure 2A). The contribution of the presumed 'silent' allele varies from 31% to 49% of the total IGF2 mRNA expression, indicative of almost equal activation of both alleles.

### IGF2 gene expression levels in relation to imprinting status

To investigate whether LOI of *IGF2* is related to the high expression of IGF2 we compared the IGF2 mRNA expression levels with the LOI/ROI status of the RA FLS. This analysis revealed that the IGF2<sup>high</sup> RA FLS are associated with LOI of *IGF2* ( $p=0.02$ ; figure 2B). These results indicate that increased expression of IGF2 in a subset of RA FLS is a consequence of the LOI status of the presumed 'silent' *IGF2* allele, leading to biallelic expression.



**Figure 2** Insulin-like growth factor 2 (*IGF2*) gene expression levels according to *IGF2* imprinting status in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS). (A) This picture shows an allele-specific transcript quantification (ASTQ) analysis for 10 representative RA FLS samples, which are heterozygous for the *Apal/AvalI* polymorphism (lanes 1–10). After *AvalI* digestion, real-time (RT)–PCR products were resolved on a 3% agarose gel. We observed three different products: an undigested (214 bp) and two digested products (151 bp and 63 bp) (the 63 bp product is not shown). The smallest product is not shown in the picture. We included three genomic DNA controls, one sample homozygous for the undigested allele (lane 11), one sample heterozygous for the *Apal/AvalI* polymorphism (lane 12) and one sample homozygous for the digested allele (lane 13). Relative levels of the 214 bp and 151 bp products were determined. NA, not applicable; LOI, loss of imprinting, ROI, retention of imprinting. (B) This graph shows the comparison between the groups of RA FLS with biallelic (*IGF2* LOI) and RA FLS with monoallelic expression of IGF2 (*IGF2* ROI) in relation to their IGF2 mRNA expression level. The y-axis represents the levels of gene expression of IGF2 relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Every spot depicts a single sample and the lines represent the mean of the IGF2 expression of each group (unpaired Student's *t* test with Welch's correction,  $p=0.02$ ).

### DISCUSSION

In RA synovium, IGF2 mRNA expression in the lining cells and infiltrating mononuclear cells has been detected.<sup>12</sup> Möller *et al*<sup>13</sup> reported that synovial mononuclear cells in RA revealed maintenance of the *IGF2* imprinted status. In this study, we demonstrate that IGF2 contributes to FLS proliferation. Moreover, we observed LOI of *IGF2* in a subset of rheumatoid FLS, associated with increased IGF2 expression. LOI-associated increased expression of IGF2 has been observed in various cancer types, in which high expression of IGF2 was related to increased cell proliferation.<sup>4,6,14</sup> Our data thus suggest that LOI in rheumatoid FLS increases *IGF2* gene expression and may promote cell proliferation, contributing to FLS hyperplasia.

The distinction in IGF2 high and low expressing FLS may indicate the presence of distinct pathological mechanisms that contribute to RA.<sup>15</sup> Firestein and Zvaifler<sup>15</sup> proposed a model wherein a T-cell-dependent process might progress to a T-cell-independent process that is focused on the autonomous role of FLS. As our data suggest that the increased IGF2 expression in RA FLS is associated with low inflammatory synovium,<sup>3</sup> it is tempting to speculate that the high expression of IGF2 due to LOI contributes to the immune-independent growth of FLS. Therefore, blockade of IGF2 may correct growth abnormalities of RA FLS, suggesting that therapeutic approaches that target IGF2 or its regulators may have beneficial effects in low inflammatory RA.

ASTQ experiments showed that the contribution of the presumed 'silent' *IGF2* allele varies among FLS. Accordingly, evidence is available to indicate that rheumatoid synovial lining FLS constitute a heterogeneous population. This variation most likely reflects that not all cells in the FLS culture contribute to the LOI of *IGF2*, indicating that not all FLS within the synovial membrane have lost the imprinting of *IGF2*.

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**Competing interests** None.

**Patient consent** Obtained.

**Ethics approval** The Medical Ethics Committee approved this study.

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