Functional characterisation of ADP ribosylaysion factor-like protein 15 in rheumatoid arthritis synovial fibroblasts

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Abstract

Objective

ARL15 is a novel susceptibility gene identified in a recent GWAS in a north Indian rheumatoid arthritis (RA) cohort. However, the role of ARL15 or ARF family genes in RA aetiology remains unknown. Therefore, we aimed to i) establish the expression of ARL15 in rheumatoid arthritis synovial fibroblasts (RASF) and ii) its functional characterisation by assessing its effects on major inflammatory cytokines and interacting partners using a knockdown approach.

Methods

RASF were cultured from synovial tissue obtained from RA patients (n=5) and osteoarthritis (OA) patients (n=3) serving as controls. Expression of ARL15, ARF1 and ARF6 in RASF was checked by semi-quantitative PCR and western blots; and altered expression of ARL15, if any, by induction of RASF with TNF using real-time PCR. The effect of ARL15 on the expression of adiponectin, adiponectin receptor I, IL6 and GAPDH and on cell mobility by invasion and migration assays were assessed by siRNA mediated gene knockdown.

Results

Expression of ARL15, ARF1 and ARF6 was confirmed in RASF and OASF samples but ARL15 expression remained unaltered on TNF induction. Notably, ARL15 knockdown resulted in downregulation of IL6 and GAPDH, upregulation of adiponectin and adiponectin receptor I genes; and significant reduction in migration and invasion of RASF. Genemania showed significant interactions of ARL15 with genes responsible for insulin resistance and phospholipase D.

Conclusion

This first report on ARL15 expression in RASF and its likely role in inflammation and metabolic syndromes through a TNF independent pathway, encourages hypothesis-free studies to identify additional pathways underlying RA disease biology.

Key words

rheumatoid arthritis, synovial fibroblasts, ARL15

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Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory condition that affects ~1% population globally but with a marked increase in the incidence of disease among women (1, 2). This disease is characterised by chronic inflammation of the synovial membrane resulting in the damage of mainly the smaller joints and shows a wide variety of other clinical symptoms. Rheumatoid arthritis synovial fibroblast (RASFs) are the most aggressive cells found in the synovium which secrete many inflammatory cytokines and Matrix metalloproteinases (MMPs) resulting in the destruction of bone and cartilage, with invasion and migration being the key features (3). This property of RASF is dependent on external as well as local factors such as invasiveness which has been detected by *in vivo* assays using RASF cartilage co-implantation (4). RA is marked by an abnormal production of cytokines, with major ones such as TNF and IL6 consequently being targeted for effective therapy. TNF plays a central role by stimulating the production of other cytokines and increasing the expression of many other adhesion molecules and MMPs. IL6 on the other hand, has been shown to be present in the synovial fluid and is produced by many cell types including RASF and chondrocytes but its production in higher amount is shown to be due to the stimulation by TNF (5).

Several studies suggest that RA is controlled by a combination of different genetic and environmental factors (6). While non-genetic factors remain elusive, a large number of genes/loci have been identified to confer susceptibility to RA (7). Of these, HLA genes have been confirmed to be the major risk determinants and association of *HLA-DR4* with disease has been established across several populations (8-11). More recently, genome-wide association studies (GWAS) of RA in Caucasian populations and their meta analysis have identified >100 genes, but their contribution towards understanding the disease mechanism and designing effective treatment still remains limited (12). Further, total disease heritability also remains to be explained and therefore, transethnic studies have been suggested to be insightful. In such a cross ethnicity replication effort carried out in our laboratory, 47 of these loci (44 European and 3 Asian specific) reported in a meta-analysis (13) were tested for association in a modest sized RA cohort from north India. Limited replication of only seven genes and nine loci of which only two (both from HLA DRB1 locus) withstood Bonferroni correction were observed (14) warranting an independent GWAS in this ethnically distinct Indian population. In the first ever GWAS which followed, we identified a novel association (p=6.57E-06) of a variant (C>A, rs255758) in a non HLA gene namely *ARL15* (5p15.2) with RA in a north Indian cohort. We further demonstrated that this risk variant was correlated with higher amounts of adiponectin levels in RA patients (15). Another variant (rs4311394) in *ARL15* has previously been shown to influence levels of adiponectin among type 2 diabetes patients (16). Association of RA with metabolic and cardiovascular risk is well known (17). Adiponectin is a protein hormone and a cytokine regulating fatty acid oxidation. This cytokine in combination with leptin has been shown to reverse insulin resistance in mice (18-19) and thus its inhibition is expected to increase metabolic risk. Conversely, expression of *ARL15* in insulin responsive tissues, including adipose tissue with highest expression in skeletal muscle, the main site of insulin mediated glucose disposal but which does not synthesise adiponectin has been reported (15). Based on this, the authors concluded that *ARL15* is a good candidate to be involved in cellular insulin resistance. This has been confirmed in a recent study with insulin induction in HEK cells which have been modelled to mimic T2D (20).

ARL 15 is a small G protein belonging to ADP ribosyl factor (ARF) family (21). ARF proteins have been shown to be involved in IL1β signalling pathway of inflammation which is independent of NFκB pathway. The inhibition of these proteins in the collagen-induced arthritis (CIA) model of RA has been shown to improve the arthritis score and inflammation (22). However, expres-

sion and/or role of either *ARL15* or any other ARF genes in RASF, a major cell type in RA aetiology, has never been reported. Based on this background, the present study aimed at functional characterisation of *ARL15*, the novel RA GWAS hit identified in our previous study (15) in RASF. We assessed its expression and attempted to understand the likely mode by which it affects inflammation in RA by expression profiling its known interacting partners (Adiponectin, GAPDH) and major inflammatory cytokines (TNF and IL6) combined with a knockdown approach.

Materials and methods

Recruitment of study subjects

This study was carried out after obtaining institutional ethics committee clearance from All India Institute of Medical Sciences (AIIMS) and University of Delhi South Campus (UDSC), New Delhi. This study was conducted in accordance with all relevant protocols. RA patients were diagnosed in accordance with the revised American College of Rheumatology/European League Against Rheumatism criteria 2010 (by UK, Department of Rheumatology, AIIMS). Synovial tissue and blood samples from five RA (RA4, RA6, RA7, RA8, RA10) and three Osteoarthritis (OA2, OA3 and OA4) patients undergoing total knee replacement surgery at AIIMS, New Delhi were collected with informed consent. DNA was isolated from venous blood samples using routine phenol chloroform extraction method. After qualitative and quantitative analysis, DNA was stored for future genetic analysis.

Generation of patient derived synovial fibroblast cell lines (RASF)

Synovial fibroblasts from synovial tissue of five RA and three OA patients were cultured as described elsewhere (23). Briefly, synovial tissue (approximately 3–4 grams) was first minced and then incubated with collagenase (C2139, SIGMA, USA) in DMEM (1199504, GIBCO, USA) overnight at 37°C. Collagenase containing medium was removed by centrifugation at 1200 rpm for 15'. The resulting pellet was suspended in complete medium (with 10% fetal bovine serum) and seeded into atleast two T-25 flasks. Cells were grown at 37°C and sub-passaging of synovial tissue derived fibroblasts (RASF) was done as needed and aliquots after each sub-passage were frozen for future use. RASF from flasks with ~80% confluence after third passage were used for all the experiments.

Test of homogeneity of RASF

RASF cultured as detailed above and which were in 3rd passage were analysed by fluorescence activated cell sorter (FACS) using antibody against cell surface markers for fibroblast (CD90thy1 FITC, A16371 Invitrogen, USA), matched isotype (IgG1 FITC, BioLegends, USA) and untreated cells served as controls. RASF with ~80% confluence were trypsinised to bring them to suspension followed by blocking with 3% BSA in PBS for 30' at room temperature (RT). Cells were then incubated with CD90thy1 antibody and isotype controls for the respective analysis at 37°C in the dark for 60' followed by two PBST (PBS with 0.1% Tween 20) washes. Cells were then suspended in PBS and taken for FACS analysis in a BD FACS Calibur multicolour flow cytometer (BD Biosciences, USA) at the central instrumentation facility (CIF), UDSC. This was done for all five RA and three OA samples included in this study.

Gene expression of ARL15 *and other related genes by RT-PCR*

Fibroblasts were harvested following routine trypsinisation protocol and total RNA was isolated using routine trizol (TRI reagent, T9424, SIGMA, USA) method.1μg of total isolated RNA was reverse transcribed using cDNA synthesis kit (Verso cDNA kit, AB1453A, Thermofisher, USA) and genomic DNA contamination was removed as per manufacturer's protocol. Expression of *ARL15,* and other candidate genes including two *ARF* genes namely *ARF1* and *ARF6* were checked using gene specific primers following standard protocols. Actin or 18s RNA served as controls for all the PCR reactions along with the genes of interest. All the experiments described below were done in triplicate and in three different RA (RA4,RA6, RA10) samples from one T25 flask each for each of the samples.

Detection of ARL15 by western blot

Cell lysates of harvested cells were prepared using RIPA buffer for western blot analysis. RASF were washed with PBS and then incubated with RIPA buffer for 5'. Cells were kept on ice for 30' and vortexed periodically before centrifugation at 12000 rpm for 30'. Protein estimation in RASF lysates were performed using BCA kit (23227, BCA protein assay kit, Thermo Scientific, USA). Equal amount of lysates were loaded onto a 12% SDS PAGE followed by overnight transfer on to PVDF membrane. Membrane was blocked using 5% BSA for one hour at RT and then incubated with primary antibody against ARL15 (ab 106441, Abcam, USA) or Actin (ab8227, Abcam, USA). Membrane was washed with PBST and then incubated with HRP conjugated secondary antibody (ab97200, Abcam, USA) and washed again before developing the blots using chemilumiscence kit (34077, Super signal west pico, Thermofisher, USA).

RASF induction with TNF

RASF obtained on trypsinisation as detailed earlier were plated at a density of 1–1.5x10^6 in six well plates. Cells were incubated in serum and antibiotic free media overnight prior to TNF induction. Cells were then induced with zero, 10ng/ml and 20ng/ml TNF-α (PHC3015, Thermofisher, USA) for 24 hours and harvested for RNA isolation. Expression of *ARL15, IL-6* and *18s* was assessed by Real-time PCR (using Quantstudio 6, Thermofisher, USA).

Knockdown of ARL15 *using siRNA*

SiRNA for *ARL15* was custom synthesised and silencer select scrambled siRNA (4390843, Ambions, Invitrogen, USA) to be used as control were procured. RASF obtained as detailed above were seeded at the density of 1–1.5x10^6 in six well plates. 24 hours prior to transfection, cells were incubated in serum and antibiotic free media. RASF culture with ~80% confluence was transfected with 10nM of *ARL15*

siRNA using lipofectamine-2000 (11668500, Invitrogen, USA). Cultures transfected with 10nM of scrambled siRNA and lipofectamine only were used as siRNA and transfection controls respectively. Six hours later the media in the flasks were replaced with complete media. The 2nd round of siRNA transfections were carried out after 24 hours and cells were harvested after 24–36 hours.

Invasion and migration assays

The effect of *ARL15* knock down on the invasion and migration ability of RASF was checked using cell inserts of 8 micron (μm) size (3422, Corning, USA). Matrigel (356234, Corning, USA) was coated on the inserts and kept at 37°C for one hour. Cells were trypsinised and seeded at the density of $5x10^4$ on the matrigel coated cell inserts and incubated for 12 hours at 37°C. Cell inserts were then taken out and matrigel along with remaining cells were removed using cotton swab followed by cutting of membrane containing invaded cells. These cells were stained with DAPI (S36942, Thermofisher, USA) and incubated in the dark for one hour at RT following which number of invaded cells were counted at six different locations under a fluorescence microscope (Zeiss, Germany).

Wound healing assay was done to check the migration ability of RASF after *ARL15* knock down as described elsewhere (24). Briefly, $1\text{-}1.5x10^6$ cells were seeded into six well plates and when ~80% confluency was reached, linear scratch was made using sterile pipette tips. This was followed by transfections with *ARL15* and scrambled siRNAs respectively and grown at 37°C for 18 hours. These experiments had two technical replicates each and three biological replicates (RA4, RA6 and RA10). Phase contrast images were captured at zero and 18 hours after wound creation.

Statistical analysis

Statistical differences between each of the *ARL15* knockdown and control experiments as described above were calculated with the Student's *t*-test or a one-way and two-way ANOVA using Graph Pad Prism 5 (GraphPad Software, USA). $p<0.05$ was considered significant.

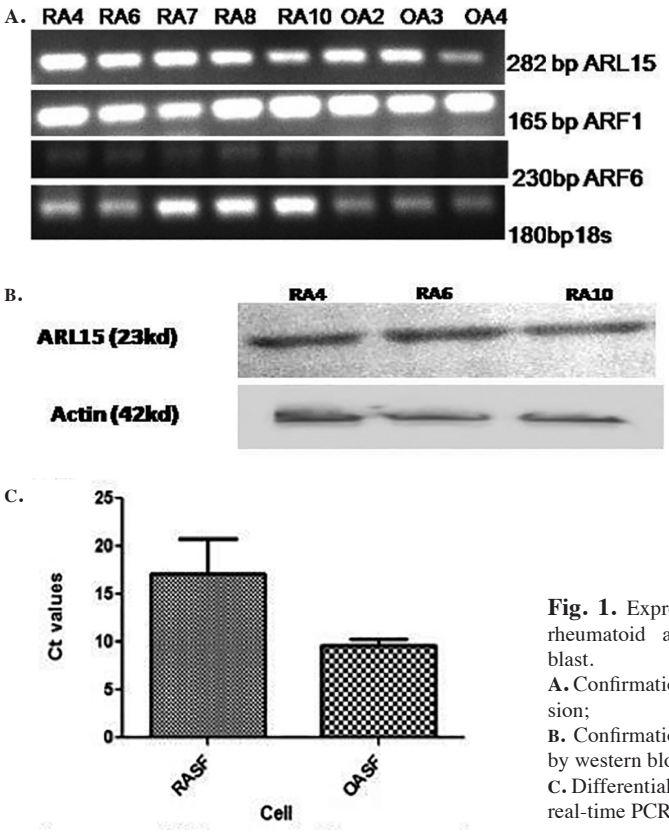


Fig. 1. Expression of ARF genes in rheumatoid arthritis synovial fibroblast.
A. Confirmation of ARF genes expression;
B. Confirmation of *ARL15* expression by western blot;
C. Differential expression of *ARL15* by real-time PCR.

Results

RASF confirmation using FACS

The FACS experiments confirmed up to ~99.9% CD90thy1 positive RASF after 3rd passage. Cells beyond 3rd passage were used for all the experiments (Supplementary Fig. 1).

ARL15 *is expressed in RASF*

Semi-quantitative PCR using *ARL15* specific primers using RNA isolated from five independent RASF lines derived from RA patients confirmed the expression of *ARL15* (Fig. 1A). This novel observation was independently confirmed with western blot using the cell lysates from RASF (Fig. 1B). *ARL15* expression in OASF from three OA patients who were used as controls was also confirmed by semi-quantitative PCR (Fig. 1A) and western blot (Fig. 1B). There was no significant difference ($p>0.05$) in the expression levels between RASF and OASF samples as indicated by qPCR (Fig. 1C).

Further expression of two ARF family genes namely *ARF1* and *ARF6* in RASF was also confirmed by semi quantitative PCR using gene specific primers (Fig. 1A).

ARL15 expression is independent of TNF concentration

Induction of RASF with TNF did not affect the expression of *ARL15*. Expression levels remained unchanged at zero, 10 and 20ng/ml TNF concentrations (Fig. 2A). On the other hand, *IL-6* which was used as an induction control in RASF showed enhanced expression (Fig. 2A).

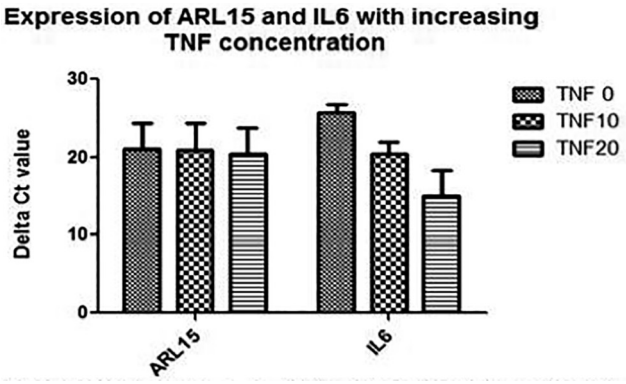
Knockdown experiments with ARL15 siRNA in RASF

Knockdown of siRNA was observed using semi quantitative PCR (Fig. 3A) and western blot (Fig. 3B). Real-time PCR experiments showed up to 4-fold knockdown of *ARL15* as compared to controls.

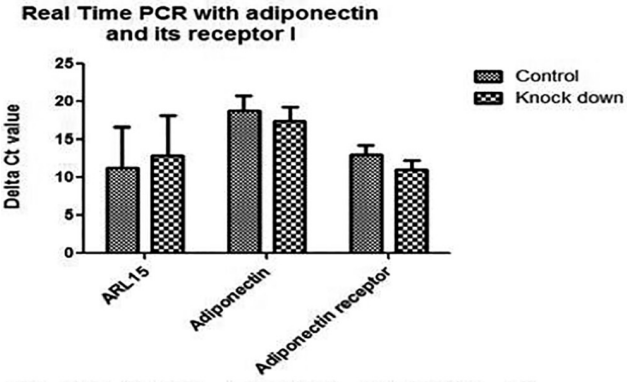
Adiponectin was up-regulated in RASF treated with ARL15 *siRNA*

Effect of *ARL15* siRNA mediated knockdown on adiponectin and its receptor type I (*ARI*) expression was in-

Fig. 2.
A. Shows change in delta Ct values for *IL6* and *ARL15* with increasing TNF concentrations by Real-time PCR in rheumatoid arthritis synovial fibroblasts.



B. Shows increased expression of *Adiponectin and Adiponectin receptor I* following siRNA mediated *ARL15* knock down in rheumatoid arthritis synovial fibroblasts.



C. Shows decreased expression of *ARL15*, *IL6* and *GAPDH* following siRNA mediated *ARL15* knock down in rheumatoid arthritis synovial fibroblasts.

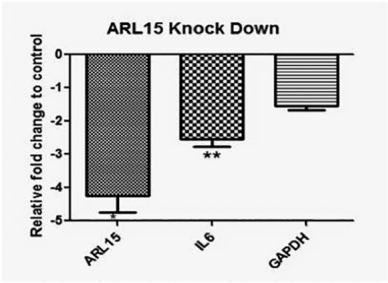
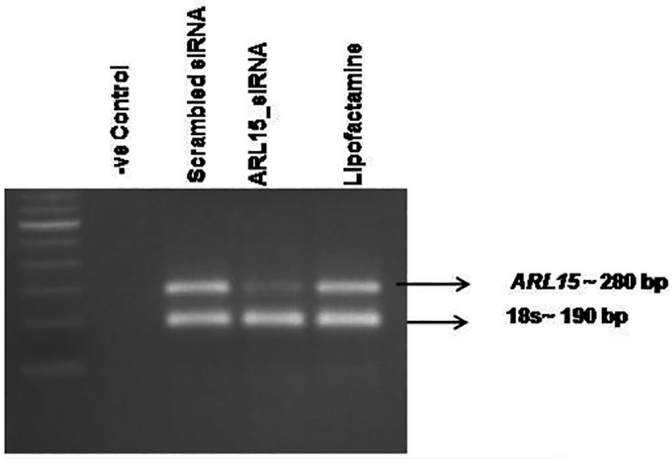


Fig. 3.
A. Semi quantitative PCR based confirmation of *ARL15* knock down in rheumatoid arthritis synovial fibroblasts.



B. Blot showing *ARL15* knock down (KD) in the rheumatoid arthritis synovial fibroblasts cell lysate.

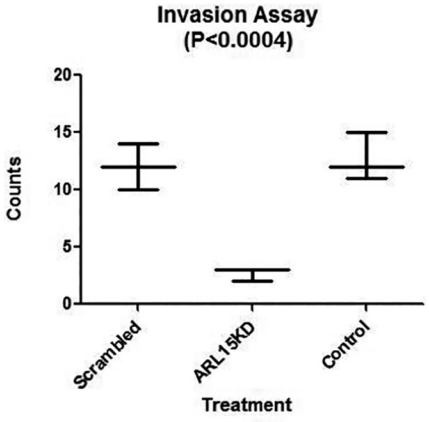


Fig. 4. Shows differences in invasiveness of rheumatoid arthritis synovial fibroblast between *ARL15* knock down, scrambled and untreated controls.

vestigated with their specific primers. Real time PCR showed that both *Adiponectin and ARI* were up-regulated in RASF treated with *ARL15* siRNA but not with scrambled siRNA and untreated control cells (Fig. 2B).

IL6 *is down regulated upon ARL15 siRNA treatment*

On the other hand, Real-time PCR showed decreased expression of *IL6* in *ARL15* knock down cells as compared to the wild type RASF (Fig. 2C). This is in contrast to the already reported up regulation of IL6 by adiponectin induction (Discussed in next section).

ARL15 knock down decreases GAPDH expression

RASF treated with *ARL15* siRNA and scrambled control siRNA were investigated for the expression of *GAPDH* using gene specific primers. Down-regulation of *GAPDH* was observed in the RASF treated with *ARL15* siRNA as compared to scrambled siRNA control (Fig. 2C)

Knockdown of ARL15 decreases invasion and migration of RASF

The invaded RASF cells were counted using Apotome image analyser (Zeiss, Germany) on a glass slide. It was found that the knock down of *ARL15* decreased RASF invasion through the matrigel (Fig. 4 and Supplementary Fig. 2).

Similarly, the number of migrated cells which were captured under a bright

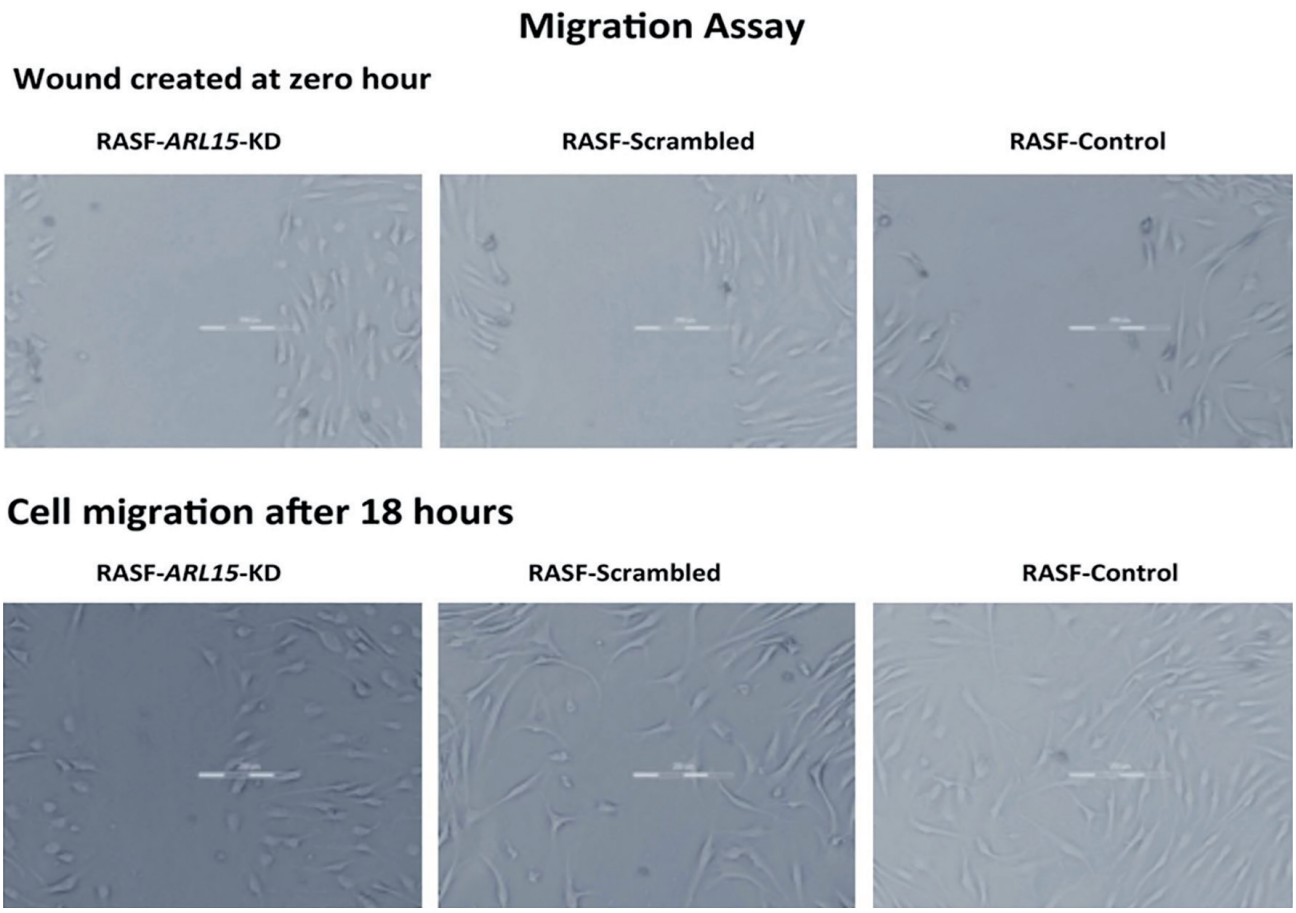


Fig. 5. Shows differences in migration of rheumatoid arthritis synovial fibroblast between *ARL15* Knock down (KD), scrambled and untreated controls.

field inverted microscope, also showed decreased rate of migration in RASF treated with *ARL15* siRNA as compared with those treated with scrambled siRNA and untreated control (Fig. 5).

Discussion

Functional studies of novel risk gene findings from the contemporary hypothesis free GWAS approach for a wide range of common complex traits can help identify unknown biological mediators of disease and may thus provide potential therapeutic targets. However, only very few susceptibility genes have been validated functionally. This study was mainly focused on the functional analysis of *ARL15*, a novel susceptibility gene identified in RA GWAS in a north Indian population carried out previously in our laboratory (15). *ARL15* belongs to the small GTPase super family of ARF proteins. ARFs are further divided into ARF and ARF like genes (ARL) which are structurally similar to ARFs (21) but their molecular and physiological functions are largely undetermined (25). This is the first ever report on the functional relevance of any ARL gene in RASF to the best of our knowledge. Based on our previous work, risk variant (rs255758) in *ARL15* was shown to alter the level of adiponectin (15), a cytokine with probable role in RA biology. In this study, we established for the first time that *ARL15* expresses in RASF, the most destructive cell type involved in RA; further, we also confirmed *ARL15* expression in OASF (Fig. 1A). No change in expression of *ARL15* levels in RASF upon TNF induction (Fig. 2A) suggests a functional role of *ARL15* independent of TNF pathway. This is in agreement with previous findings of lowered severity in CIA mouse models following ARF inhibition but without any change in TNF levels (22). Further, in the same study regulation of IL1β was shown to be independent of NFκB pathway suggestive of alternate pathways in the aetiology of RA.

In order to get more insights into *ARL15* function, siRNA mediated knock down of *ARL15* was carried out and expression profiles of several known interacting partners of *ARL15* were analyzed in this study. As mentioned in the introduction, RA is associated with markedly enhanced metabolic and cardiovascular risk and adiponectin inhibition is expected to further enhance metabolic risk (17). In order to understand the adiponectin interaction with *ARL15*, we checked the expression of adiponectin and *ARI* in RASF on *ARL15* knockdown. Adiponectin and *ARI* levels were both higher as compared to RASF treated with scrambled siRNA controls (Fig. 3B). We had in our previous study shown increased levels of adiponectin in RA patients with the risk genotype (15) (AA, rs255758) of *ARL15* which lends support to our present observations. Taken together this may imply the role of *ARL15* in metabolic syndromes. On the other hand, *IL6* expression was decreased on *ARL15* knockdown (Fig.

3C), suggesting its independent effect on the inflammatory pathway mediated by *IL6*. This hitherto unreported role of *ARL15* via adiponectin and/or *IL6* in RA disease biology warrants discussion. The correlation between adiponectin and *IL6* has been controversial. Based on a few *in vitro* studies, *IL6* expression has been reported to increase on induction with adiponectin via TNF pathway in RASF but few studies show that *IL6* expression is inhibited by adiponectin in human macrophage and leucocytes (26). However in CIA mouse model, an anti inflammatory effect of adiponectin with decrease in *TNF, IL1β* and *MMP3* but with increased *IL6* level has been reported (27), which suggests that *IL6* expression is independent of TNF pathway. Thus the expression of *IL6* seems to be tissue specific. Our experiments showing decreased *IL6* but increased adiponectin levels on *ARL15* knockdown and unaltered levels of *ARL15* but increased *IL6* levels on TNF induction supports the CIA mouse model findings and reiterates a possible alternate control of IL6 and consequently its possible additional role(s) in different disease phenotypes such as *IL6* in insulin resistance. It may be mentioned here that in a recent replication study of 64 SNPs in an independent European RA cohort, *IL6* was reported as a likely novel RA associated locus (28). With this knowledge, the role of *ARL15* in RA may be interpreted as follows.

Adiponectin has long been shown to be responsible for insulin sensitivity in type II diabetes (T2D) (19) and as mentioned earlier *ARL15* has also been shown to influence adiponectin levels in T2D (16). *IL6* like adiponectin is very widely known to be produced from fat cells and involved in insulin resistance (29). Interestingly, one of the common features between RA and T2D is insulin resistance (16, 17) and our observation of increased adiponectin and decreased *IL6* expression after *ARL15* knock down (Fig. 3C) suggests that *ARL15* might be linked via insulin resistance in RA patients. This is similar to its previously reported role in T2D (16, 20) and more importantly, provides yet another evidence of association of RA with metabolic risk (17).

Finally this derives additional support from the interaction pattern of *ARL15* observed in Genemania (genemania.org) wherein it was seen to interact directly with *GAPDH, MTOR* and *RHEB* (Supplementary Fig. 3) which are all involved in glucose metabolism and insulin resistance (30). In addition, phospholipase D (PLD) was also seen to have direct interaction with ARL15 at both gene and protein levels (Supplementary Fig. 3). This we speculate could be the most direct effect of *ARL15* in RA, since PLD is well known to be upregulated in RA and it also regulates IL15 levels leading to osteoclastogenesis (31). A correlation between IL6 and IL15 receptors based on synovial fluid ELISA of RA patients has already been reported (32). ARL15 is structurally similar to ADP-ribosylation factors and Ras-related GTP-binding proteins which play key roles in the regulation of intracellular vesicle trafficking (16). The vesicle trafficking has been shown to be the messenger of immune responses and it also plays a bigger role in the migration and invasion of cells (33). Moreover, PLD activity is required for the invasive and migratory property of cells (34). Taken together these findings, lower invasion and migration of RASF following *ARL15* knock down (Figs. 4, 5 and Supplementary Fig. 2) lends further support to the likely role of *ARL15* in RA biology.

In summary, the results of this study based on investigations of known interaction partners suggest that *ARL15* might be involved in down regulation of i) the non-TNF mediated inflammatory parameters in RASF and ii) the metabolic pathway via insulin resistance mediated by adiponectin and PLD leading to altered invasion/migration potential of RASF, both with therapeutic relevance. Hypothesis-free assessment of effects of *ARL15* knock down in RASF may provide further insights into disease-related pathways.

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