Inflammation and apoptosis in the synovium of patients with rheumatoid arthritis
the role of SIRT1 and microRNA-34a*

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INFLAMMATION AND APOPTOSIS IN THE SYNOVIUM OF
PATIENTS WITH RHEUMATOID ARTHRITIS
- THE ROLE OF SIRT1 AND microRNA-34A*

A dissertation submitted to
ETH ZURICH

for the degree of
DOCTOR OF SCIENCES

presented by
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2012
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SUMMARY

Tying shoes, climbing steps and brushing hair – such simple tasks of daily life are for thousands of patients severely suffering from rheumatoid arthritis impossible to perform. Arthritic joints are stiff in the morning, swollen, painful and inflamed. With time, the articular cartilage and adjacent bone gets irreversibly destroyed, resulting in disability of the patient. The symptoms are caused by a misguided immune system that attacks healthy tissue of the body. Despite enormous efforts in research and development of new therapies, the initial trigger of rheumatoid arthritis is still unknown and until now, no cure exists.

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic joint inflammation and progressive destruction of cartilage and bone leading to severe joint pain and ultimately loss of function. The resident cells of the hyperplastic synovium in rheumatoid joints are synovial fibroblasts (RASF) and macrophages. Activated RASF display a highly aggressive phenotype and contribute to chronic joint inflammation by their production of proinflammatory cytokines and cartilage degrading enzymes. The resistance to apoptosis is a key feature of RASF as it results in the characteristic hyperplasia of the synovial tissue seen in the RA joint. Furthermore, synovial macrophages produce excessive amounts of proinflammatory cytokines, thus propagating inflammation. Both RASF and macrophages/monocytes in RA show a complex pattern of intrinsic molecular activation. The underlying mechanisms of this phenotype are incompletely known.

In recent years, sirtuins (SIRT) raised considerable interest since they were shown to influence important cellular functions such as longevity and inflammation. SIRTs are a family of NAD+-dependent histone deacetylases that target not only histones, but also numerous transcription factors and coregulators. Since both apoptosis and inflammation pathways are altered in RA, we hypothesized that SIRTs might be involved in the development of the activated and invasive phenotype seen in synovial cells from patients with RA.

In this work, it is demonstrated that RASF and monocytes in RA express SIRT1, 2, 3, 4, 6 and 7. SIRT1 was among the most abundantly expressed sirtuins in RASF. SIRT1 expression was found to be constitutively higher in synovial tissues and cells from RA compared to non-inflammatory osteoarthritis (OA) patients. Stimulation of RASF with TNF-α further induced the production of SIRT1. Overexpression of SIRT1 conferred resistance to apoptosis in RASF. This anti-apoptotic effect prolonging the life span of RASF is crucial, since SIRT1 was found to directly enhance proinflammatory cytokine and chemokine expression in
synovial cells, thereby contributing to chronic joint inflammation. Thus, we demonstrate an anti-apoptotic and proinflammatory role of SIRT1 in the pathogenesis of RA.

As synovial fibroblasts are involved in the propagation of inflammation, modulation of apoptotic pathways may be of considerable therapeutic interest. Therefore the microRNA (miR) dependent regulation of anti-apoptotic SIRT1 expression was studied. The small non-coding miRs function as posttranscriptional modulators of gene expression, estimated to regulate about 30% of the human genome. Recently, an aberrant miR expression pattern in RA synovial cells was reported. In particular, a screening assay suggested down regulation of the family of miR-34 in SF from patients with RA compared to OA. Existing data reported that this miR family is involved in the regulation of apoptotic pathways via targeting SIRT1. In this work, it was confirmed that miR-34a regulates the expression of SIRT1 in RASF.

Furthermore, we demonstrate that basal expression of the passenger strand of miR-34a, miR-34a*, is reduced in RASF compared to OASF. We identified the promoter of miR-34a/34a* to be methylated in RASF and showed that transcription of the miR-34a duplex is induced upon treatment with demethylating agents. In this work, we demonstrate that miR-34a* binds to the 3'UTR of the apoptosis inhibitor XIAP (X-linked inhibitor of apoptosis protein), a novel identified target of miR-34a*, and thus blocks the protein biosynthesis of XIAP. In accordance, enforced expression of miR-34a* resulted in an increased rate of FasL and TRAIL induced apoptosis, whereas silencing of miR-34a* mediated apoptosis resistance in RASF. Since the role of miRs in inflammatory arthritis is only beginning to be explored, Chapter III of this thesis aims to elucidate miR dependent pathways involved in the apoptosis resistance of RASF as this may lead to new strategies to optimize treatment of this debilitating disease.
ZUSAMMENFASSUNG

Schuhe schnüren, Treppen steigen und Haare kämmen – diese einfachen Alltagsbewegungen sind für Tausende von Menschen, die schwer an der rheumatoiden Arthritis (RA) leiden, unmöglich auszuführen. Die betroffenen Gelenke sind charakterisiert durch Morgensteifigkeit, Schwellung, Schmerz und Entzündung. Mit voranschreitender Krankheit werden Gelenksknorpel und benachbarter Knochen irreversibel zerstört, bis hin zur völligen Bewegungsunfähigkeit des Patienten.

Diese Symptome werden verursacht durch ein falsch gesteuertes Immunsystem, das gesundes Gewebe angreift. Trotz enormen Bemühungen in der Erforschung der Krankheit wie auch in der Entwicklung neuer therapeutischer Möglichkeiten bleibt die Ursache von RA unbekannt und damit die Krankheit unheilbar.


In der vorliegenden Arbeit wird gezeigt, dass RASF SIRT1, 2, 3, 4, 6 und 7 besitzen. Dabei war SIRT1 eines der am höchsten exprimierten Sirtuine. Im Synovialgewebe von RA-Patienten konnten signifikant höhere Konzentrationen an SIRT1 im Vergleich zu Patienten mit nicht-entzündlicher Osteoarthritis (OA) festgestellt werden. Stimulierung von RASF mit TNF-α führte zu einer weiteren Induktion von SIRT1. Desweiteren wird im Kapitel II gezeigt, dass SIRT1 die Synovialzellen vor Apoptose schützt und überdies die Produktion von proinflammatorischen Zytokinen und Chemokinen erhöht.

Da die synovialen Fibroblasten eine Schlüsselrolle in der Pathogenese der rheumatoiden Arthritis einnehmen, sollte ein besonderes Augenmerk auf die Ursachen, die zu deren Apoptose-Resistenz führen, gelegt werden. Aus diesem Grund wurde die Regulation von anti-apoptotischem SIRT1 mit Einbezug von MicroRNA (miR) Analysen genauer studiert. MiRs sind post-transkriptionelle Modulatoren der Genexpression. Es wird vermutet, dass rund 30 % des menschlichen Genoms durch miRs reguliert wird. Erst kürzlich erschien aus unserem Labor eine Studie, die Änderungen im Expressionsmuster von miRs in RASF zeigt. Dabei wurden Hinweise geliefert, dass auch miR-34a, eine microRNA die bereits öfters mit Apoptosemodulierung via SIRT1 in Verbindung gebracht wurde, fehlreguliert ist. In der vorliegenden Arbeit bestätigen wir frühere Studien und zeigen, dass miR-34a die Expression von SIRT1 reguliert. Ausserdem bemerkten wir eine enorme Reduktion in der Produktion des komplementären Strangs von miR-34a, der miR-34a*, in RASF. Durch Demethylierung der Synovialfibroblasten mit entsprechenden Agenzien konnte die Transkription des miR-34a Duplex induziert werden. Im Kapitel III wird gezeigt, dass die tiefe Expression von miR-34a* mit dem apoptose-resistenten Phänotyp von RASF in Verbindung steht. Durch Aufklärung des genauen Wirkungsmechanismus wurde das direkte Zielmolekül von miR-34a*, der Apoptose Inhibitor XIAP (X-linked inhibitor of apoptosis protein), identifiziert. Verstärkte Expression von miR-34a* resultierte in einer Erhöhung der Anzahl apoptotischer Zellen nach Stimulierung mit FasL oder TRAIL, während Stilllegung von miR-34a* den Synovialfibroblasten eine Resistenz gegenüber Apoptose vermittelte.

Obwohl die Rolle von miRs in der rheumatoiden Arthritis erst gründlich erforscht werden muss, gibt es bereits jetzt viele Hinweise die auf eine wichtige Funktion in der Pathogenese deuten und damit neue Strategien zur Therapie oder gar Heilung der RA eröffnen.
CHAPTER I INTRODUCTION
**RHEUMATOID ARTHRITIS**

Rheumatoid arthritis (RA) is a systemic autoimmune disorder manifesting with chronic joint inflammation and severe pain. RA goes along with irreversible destruction of bone and cartilage, ultimately resulting in loss of function and disability of the patient. The major pathologic phenomena of RA are persistent inflammatory synovitis, involving peripheral joints in a symmetric distribution, and a synovial hyperplasia that result in the damage of cartilage. RA is a heterogeneous disease, with a spectrum of the clinical symptoms ranging from mild to severe and a marked variability in responsiveness to different anti-rheumatic treatments [1, 2]. Extraarticular complications are common and generally related to worse disease. Rheumatoid nodules are seen in almost one third of the patients, and every tenth patient with RA suffers from extraarticular manifestations such as rheumatoid vasculitis, Sjögren’s and pulmonary disease [3].

Despite extensive work, the etiology and pathogenesis of RA is still only partially understood. The initial triggers that cause RA are still unknown and the reason why the synovium is the primary target remains a mystery. Even though there has been considerable progress in the treatment of RA recently, the disease cannot be cured. Only about one-third of patients respond well to the first-line treatment methotrexate [4]. Even with the newest available drugs, there is still a subgroup of non-responders. Since the limited therapy of RA as well as the financial burden of the expensive treatment remains a problem, there is still a need for better therapeutic strategies.

**Epidemiology**

RA is the most common inflammatory rheumatic disease as it affects about 1 % of the population. As seen in most autoimmune diseases, RA is about three times more common in women then in men [5]. Although the disease may present at any age, the onset is most frequent in patients between 35 and 50 years of age.

RA is world widely distributed. Incidence as well as prevalence rates differ between regions. While some of North America’s indigenous peoples display high rates of rheumatic diseases, low abundance or even absence of RA was discovered in rural African populations [6-8]. Such studies suggest the involvement of genetics. However, a Finnish study reported on regional differences in the incidence of RA, which was found to be about 2.5 fold higher in north-eastern compared to north-western Finland. The authors concluded the contribution of environmental factors to the occurrence of RA [9].
Classification

The standard and accepted means of defining RA is by the use of classification criteria. Until 2010, the 1987 American College of Rheumatology (ACR) criteria were used internationally to define RA [10]. However, these criteria have been criticized for their lack of sensitivity in early disease. They were therefore refined in 2010 by the ACR/EULAR (European League Against Rheumatisms). Classification as “definite RA” is based on the confirmed presence of synovitis in at least 1 joint, absence of an alternative diagnosis that better explains the synovitis, and achievement of a total score of 6 or greater of a possible of 10 (Table 1) [11]. The cells used for the experimental work described in the thesis were derived from patients that fulfilled the 1987 ACR criteria.

<table>
<thead>
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<th>Target population (Who should be tested?): Patients who:</th>
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<tr>
<td>1) have at least 1 joint with definite clinical synovitis (swelling)</td>
</tr>
<tr>
<td>2) with the synovitis not better explained by another disease</td>
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<tr>
<th>Classification criteria for RA (score-base algorithm: add score of categories A-D; a score of 6/10 is needed for classification of a patient as having definite RA)</th>
<th>Score</th>
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<tr>
<td><strong>A) Joint Involvement</strong></td>
<td></td>
</tr>
<tr>
<td>1 large joint</td>
<td>0</td>
</tr>
<tr>
<td>2-10 large joints</td>
<td>1</td>
</tr>
<tr>
<td>1-3 small joints (with or without involvement of large joints)</td>
<td>2</td>
</tr>
<tr>
<td>4-10 small joints (with or without involvement of large joints)</td>
<td>3</td>
</tr>
<tr>
<td>&gt;10 joints (at least 1 small joint)</td>
<td>5</td>
</tr>
<tr>
<td><strong>B) Serology</strong> (at least 1 test result is needed for classification)</td>
<td></td>
</tr>
<tr>
<td>Negative RF and negative ACPA</td>
<td>0</td>
</tr>
<tr>
<td>Low-positive RF or low-positive ACPA</td>
<td>2</td>
</tr>
<tr>
<td>High-positive RF or high-positive ACPA</td>
<td>3</td>
</tr>
<tr>
<td><strong>C) Acute-phase reactants</strong> (at least 1 test result is needed for classification)</td>
<td></td>
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<tr>
<td>Normal CRP and normal ESR</td>
<td>0</td>
</tr>
<tr>
<td>Abnormal CRP or abnormal ESR</td>
<td>1</td>
</tr>
<tr>
<td><strong>D) Duration of symptoms</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;6 weeks</td>
<td>0</td>
</tr>
<tr>
<td>≥6 weeks</td>
<td>1</td>
</tr>
</tbody>
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Table 1: The 2010 American College of Rheumatology/European League Against Rheumatisms classification criteria for rheumatoid arthritis.

ACPA = anti-citrullinated protein antibody; RF = rheumatoid factor; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate. For a more detailed description of the criteria, please refer to Aletaha et al., Arthritis & Rheumatism, 2010.
Disease activity score in 28 joints (DAS28)

The basis for judging disease activity in clinical practice was made in 1990 by defining criteria for a *disease activity score* (DAS) [12]. Originally, the DAS score included judgment of 44 defined joints. Five years later, reduction to only 28 joints made the revised DAS28 feasible for use in daily clinical practice to monitor RA disease activity [13].

DAS28 combines the number of tender and swollen joints (out of a total of 28 defined joints), and the erythrocyte sedimentation rate (a blood marker of inflammation) into an overall assessment of RA disease activity. A DAS28 score greater than 5.1 implies active disease, less than 3.2 well controlled disease, and less than 2.6 indicates that the patient is considered to be in remission [14]. The use of a single index is advantageous because the disease activity is thus described at a specific time point and not in relative changes over a time period as the ACR response criteria do. By comparing a patient’s DAS28 score over multiple time points, one can substantiate the improvement or response to therapeutic treatments [14].

ACR response criteria

The ACR response criteria are in general use to measure improvement in RA in an individual patient. In the 1990s, the ACR20 was developed, defined as at least 20 % improvement in both tender or swollen joint count and at least 20 % improvement in 3 of the following 5 criteria: the physician’s assessment of disease activity, the patient’s assessment of disease activity, the patient’s assessment of pain, the patient’s assessment of physical function and the levels of either the C-reactive protein or the erythrocyte sedimentation rate [15]. Widespread use of the ACR20 highlighted the need for more pronounced measurements of disease improvement. Therefore, ACR50 and ACR70 response criteria were implemented, meaning an improvement of 50 and 70 % in disease activity, respectively [16].

Diagnosis

There is no singular test for the diagnosis of RA. Instead, a rheumatologist uses a combination of lab tests, imaging methods and judgements on the pattern of clinical symptoms and on the distribution of the inflamed joints to diagnose RA. The doctor examines the joints for inflammation, tenderness, swelling and deformity to evaluate the patient’s DAS28. In the lab, the patient’s blood will be tested for the presence of anti-citrullinated protein antibodies and rheumatoid factors, and the erythrocyte sedimentation rate as well as the concentration of the C-reactive protein is measured. Further, X-ray tests and MRI scanning can be used to demonstrate joint damage.
Treatment

Since the disease severity and aggressiveness differ in individual patients, the treatment of RA varies from person to person. Drug therapies for RA include analgesics, non-steroidal anti-inflammatory drugs (NSAIDs) and disease-modifying anti-rheumatic drugs (DMARDs). Whereas NSAIDs do not retard joint destruction but instead possess anti-inflammatory activity, DMARDs alter the disease process including inflammation and joint destruction. Methotrexate (MTX), an inhibitor of folate dependent enzymes, is the most commonly used DMARD and has emerged as the first-line disease-modifying drug in the treatment of RA. It has been recognized that early and aggressive treatment with DMARDs provides the best strategy to slow down disease progression. In recent years, a new generation of anti-rheumatic drugs, called biologics, significantly improved therapeutic strategies. Biologics target key cytokines such as TNF-α and IL-6 or lymphocytes and proved to be effective for the majority of RA patients. However, in spite of these promising results, there is still a subgroup of patients failing to respond to these new medication strategies. In spite of therapeutic advances that improved the lives of patients with RA, the problem is hardly solved. There is no cure for RA yet, highlighting the need for the identification of new therapeutic targets and novel predictive biomarkers.

Animal Models

In spite of great research efforts, the aetiology and pathogenesis of RA remains largely unknown. Animal models substantially contribute to the better understanding of disease mechanisms. Important criteria in the selection of the appropriate model includes similar pathology and/or pathogenesis to that of human disease, capacity to predict efficacy of agents in humans as well as the ease of use [17]. For RA, there are numerous models with histopathological features close to those of human RA. Most common animal models of RA include collagen-induced [18], antigen-induced [19] and K/BxN serum transfer-induced arthritis [20, 21] as well as the TNF transgenic mouse model [22]. The wide variety of stimuli inducing arthritis again suggests the existence of disparate aetiological pathways in RA. However, no single animal model of arthritis truly represents the human disease, but the models mimic various aspects and can be used as tools to understand particular pathways. Detailed descriptions of diverse arthritic animal models are provided in further reports [17, 23].
Aetiology

Variations in prevalence rates between specific populations within a country point to genetic components as a cause of RA, whereas the differences between specific geographical areas indicate the contribution of environmental factors. Although the cause of RA remains unknown, genetic, environmental, infectious and other so far unknown components contribute to the pathogenesis of RA [24].

Genetic factors

The most important genetic risk factor is found in the human leukocyte antigen (HLA) locus [25], accounting for 30 to 50% of overall genetic risk for RA [26]. Individuals carrying risk alleles within the HLA-DRB1 gene are more likely to develop RA [27]. These risk alleles contain a common amino acid sequence, which is important in peptide binding and therefore known as "shared epitope" [25]. Since the identification of HLA-DRB1 in 1978, it was not until 2003 that the second genetic risk factor, the peptidylarginine deiminase type 4 (PADI4), was implicated in susceptibility to RA and severity of disease [28]. However, recent studies could not observe any association of PADI4 genotype with RA in European populations [29-31]. Nowadays, numerous new genes associated with a high risk of developing RA have been identified, including PTPN22, CTLA4, STAT4 and TNFAIP3 [25].

Environmental factors

The contribution of environmental factors such as diet and smoking to the development of RA is well recognized. Supplementation of patient's diets with omega 3 fatty acids or vitamins was demonstrated to be beneficial in the treatment of RA [32, 33]. However, analysis of the role of specific dietary factors in the risk for RA is difficult, since our nutrition is of high complexity and its separation into single contributing factors nearly impossible.

The implication of smoking in the development of RA was demonstrated by numerous reports [34, 35]. Interestingly, a high susceptibility to RA was found to occur in smokers carrying the shared epitope in their HLA-DRBI gene [36], thus connecting the influences of genetics and environment in the risk for RA.

Infectious agents

Microbial organisms are hypothesized to elicit the onset of RA in predisposed people. The misguided immune system in RA is supposed to target not only pathogens but instead to attack healthy tissue and thus, an immune reaction against antigens of the patient's joints
might be induced. Such cross reaction may result in the failure of normal immunological tolerance, leading to the development of a chronic autoimmune joint disease. So far, a number of infectious agents, including human parvovirus B19, Epstein-Barr virus (EBV), Mycoplasma spp and other bacteria such as Mycobacterium tuberculosis, have been proposed as potential risk factors for RA [37]. However, no firm evidence to date has clearly defined the role of any specific infectious organism in RA. Although high antibody titres to EBV antigens have been detected in sera, synovial fluids and synovial tissues from patients with RA but not in control OA samples [38, 39], reports observing lack of evidence of EBV infection in the synovium of RA patients contradicted these findings [40, 41].
PATHOGENESIS OF RHEUMATOID ARTHRITIS

The architecture of a healthy joint enables skeletal mobility. The normal synovium encapsulates the joints and by the production of synovial fluid provides a smooth, frictionless surface for articulation. Healthy synovial tissue constitutes the main source of nutrients and signalling factors for cartilage. The synovial tissue is divided into the synovial lining and the synovial sublining layer. The sublining represents a delicate membrane, consisting of two to three cell layers mainly composed of synovial macrophages, also called Type A synoviocytes, and of synovial fibroblasts (SF), also termed fibroblast-like synoviocytes or Type B synoviocytes. In RA, the synovial membrane grows to a 10-15 cell layer thick synovial tissue. A comparison of a healthy and an arthritic joint is shown in Figure 1 [42].

The primary inflammatory site in RA is the synovium. Synovitis is associated with a hyperplasia of the synovium, due to the infiltration and subsequent proliferation of mononuclear cells including B cells, T cells and macrophages into the synovial sublining as well as the increased survival of resident cells. The hyperplastic synovial tissue, also called ńpannusó, overgrows the underlying articular cartilage and finally erodes the adjacent bone (Figure 2) [42]. The ńpannusó plays a key role in cartilage destruction in RA joints through the secretion of matrix degrading enzymes such as metalloproteinases (MMPs).

Despite many years of experimental and clinical investigations, understanding of the pathogenesis of RA is still incomplete. Since RA is a complex disease with multiple mechanisms of synovial inflammation and joint destruction, there are various hypotheses attempting to explain RA. A possible paradigm assumes the activation of innate immunity as an early step that manifests in a preclinical phase characterized by asymptomatic synovial inflammation [43]. Since Toll-like receptor (TLR) agonists like bacterial DNA and proteoglycans have been observed at high amounts in the rheumatoid synovium [24], components of the innate immune response are implicated in the disease onset. Consistent with this idea, numerous innate immune sensors such as TLRs and NOD-like receptors are found highly expressed within the arthritic joint [44, 45].

Beyond the innate immunity, interest is raised in the adaptive immune responses. The abundant expression of activated T cells and B lymphocytes has long been recognized and the success of B cell therapeutics further implicates a crucial role of the adaptive immune system in RA. Even several years before onset of the clinical signs of RA, autoreactive antibodies such as rheumatoid factors (RF) and anti-citrullinated protein antibodies (ACPA) can be detected in the patient’s blood [46, 47].
Introduction

Figure 1: Comparison of a healthy (a) and a rheumatic joint (b).
In the healthy joint (a) the synovial tissue is a two to three cell layer thick membrane that provides nutrition for the cartilage by the production of synovial fluid. In rheumatoid arthritis (b) the synovial membrane becomes hyperplastic and ultimately consists of up to 15 cell layers. Development of the synovial tissue into a hyperplastic pannus leads to migration into the articular cartilage and underlying bone. From Strand et al., Nat Rev Drug Discov, 2007.

Figure 2: Histologic comparison of synovial tissues from osteoarthritis and rheumatoid arthritis patients.
Whereas osteoarthritic synovium is only a few cell layers thick, the rheumatic synovial tissue shows hyperplasia due to cell infiltration and proliferation. In RA, synovial cells invade into cartilage at sites of invasion, leading to the destruction of the joint. Pictures are kindly provided by Dr. C. Ospelt.
Studies on anticitrulline immunity suggest the subdivision of RA into patients who are positive for ACPA and those who are ACPA negative [48]. Citrullinated peptides are derived from post-translational modifications of arginine and may result from cell damage or uncontrolled apoptosis. In addition, environmental risk factors such as smoking may induce citrullination of proteins [49]. The generation of citrullinated proteins results in production of ACPA, which are thought to be important not only in the initiation phase of RA, but are also used in the diagnosis of the disease at a later stage. Circulating ACPA might enter the joint, where they bind to the citrullinated proteins and form immune complexes, which stimulate the influx of inflammatory cells into the joint. Activated immune cells produce high amounts of pro-inflammatory cytokines that in turn recruit immune cells, thus establishing a vicious cycle that ultimately results in a breach of immune tolerance and finally leads to the development of chronic inflammatory RA (Figure 3) [50, 51]. The significance of ACPA is supported by in vivo studies, where ACPA enhanced arthritis development in mice that already had mild synovitis, indicating that these antibodies might also be pathogenic in human beings [52].

In parallel, interest in genetics is driven increasingly, since many risk loci including those found in the major histocompatibility complex (MHC), PTPN22 and CTLA4 suggest modulatory influences on adaptive immunity. A broader impact on the regulation of immune responses is assumed for STAT4 and TNFAIP3, both involved in leukocyte activation and inflammatory signalling [53].

Beside the identification of the initial trigger, a major challenge is now to define the hierarchy of all the events taking place during the progression of RA and to properly study the functional impacts of every single component.

Figure 3: A proposed model for the development of ACPA positive RA.
Citrullination of proteins is caused by yet unknown stimuli. In RA patients, an immune response to citrullinated proteins results in the production of ACPA. Recruitment of these antibodies from the circulation into the joint results in the formation of immune complexes that perpetuate the joint inflammation into chronic RA. From Klareskog et al., 2008, Annu. Rev. Immunol.
**Cellular composition of the rheumatic joint**

The inflamed synovial tissue encompasses many cell types including macrophages, fibroblasts, B cells, dendritic cells and T cells. When rheumatoid synovium is enzymatically digested *in vitro*, only the two major cell types - synovial fibroblasts and macrophages - adhere to tissue culture dishes. While synovial macrophages rarely survive more than a few weeks in culture, synovial fibroblasts can be passaged for several months *in vitro* [42].

The availability of diseased tissue from the rheumatic joint has allowed for investigation of the molecular mechanisms involved in the pathogenesis of RA. The tissue is obtained during joint replacement surgery from patients with established and usually longstanding disease. To study early events occurring at the onset of RA, biopsy material is needed.

**Synovial fibroblasts**

Synovial fibroblasts have initially been considered as quiescent cells. However, in recent years, cumulating evidence has identified these cells as active players in the pathogenesis of RA [54]. Numerous studies have documented that RASF actively contribute to joint degradation due to their production of large amounts of proinflammatory cytokines and chemokines and their active promotion of cartilage erosion by expression of high amounts of destructive enzymes.

The transition from a quiescent mesenchymal into a destructive cell has been well documented by several studies. In the early 1980s, initial description of a tumour like phenotype was made, noted as distinctive morphological features such as abundant cytoplasm, large pale nuclei and a dense, rough endoplasmatic reticulum [55]. Several years later, in 1996, RASF were functionally proven to be activated, aggressive and destructive cells. Human RASF were co-implanted with human cartilage into the severe combined immunodeficient (SCID) mice. Whereas RASF actively degraded human cartilage, control implanted synoviocytes from OA patients and normal dermal fibroblasts did not [56]. Since RASF destroyed cartilage even in the absence of cellular and humoral immune responses, the maintenance of the activation state of RASF seems to be independent of the inflammatory environment within the synovium, and therefore intrinsic and permanent.

Once activated, RASF produce increased amounts of matrix degrading enzymes, inflammatory cytokines and chemokines. The secretion of chemotactic molecules such as GCP-2 (granulocyte chemotactic protein 2), RANTES (regulated on activation, normal T-cell expressed and secreted), MCP-2 (monocyte chemotactant protein 2), CXCL12 and CXCL16 by RASF attracts leukocytes towards the synovium [57-59]. In addition, cell
migration was shown to be enhanced upon production of chemoattractants such as SDF-1 (stromal cell derived growth factor-1), MCP, MIP (macrophage inflammatory protein), RANTES and interleukin-8 [60-64].

Although several studies focused on the underlying mechanisms, the pathophysiological processes causing the permanent activation of RASF remain unknown. However, a variety of transcription factors such as c-Fos, Ras, Raf, Myc and Myb, which are typically abundant in tumour cells, are also found in RASF at high levels [42]. Additionally, inactivation of the tumour suppressor gene p53 was shown to increase disease severity and to augment joint destruction in the collagen-induced arthritis mouse model [65]. Further, the production of MMPs was associated with somatic mutations in the p53 gene [66, 67], while others reported on the induction of p53 at sites of invasion as a result of the destructive processes driven by RASF [68, 69].

Synovial fibroblasts express a variety of proteins that are unusual for mesenchymal cells, including VCAM-1 (vascular cell adhesion molecule-1), CD55, b1 integrins and cadherin-11. Adhesion molecules are considered to be crucial in the pathogenesis of RA, as they enable RASF to attach to the articular cartilage and to deeply invade into the extracellular matrix. Their importance has been shown in cadherin-11 deficient mice that displayed a hypoplastic synovial lining and were resistant to inflammatory arthritis (Figure 4) [70]. Thus, cadherin-11 is suggested to play a key role in synovial cell attachment and in determine the behaviour of synovial cells with regard to inflammation and destructive cartilage invasion.

Figure 4: Cadherin-11 modulates synovial tissue response in arthritis.
Synovial fibroblast (SF) micromass organ cultures only formed a condensed lining layer when wildtype SF (a) and not Cadherin-11 deficient SF (b) were used. From Lee et al., Science, 2007.
Once attached to cartilage and bone surface, RASF actively drive damage of cartilage, bone, and tendons by secreting matrix-degrading enzymes such as MMPs and cysteine proteases. MMPs include collagenases (MMP-1, -8, -13), stromelysins (MMP-3, -10, -11), gelatinases (MMP-2 and -9) and membrane-type MMPs, which are all expressed at low concentrations in healthy synovium but show increased abundance in the arthritic joint [71, 72]. Beside MMPs, cysteine proteases such as cathepsins K and L also contribute to matrix degradation. Cathepsin K is involved in both cartilage and bone degradation, whereas cathepsin L only degrades collagens and proteoglycans of cartilage matrix [73, 74]. Additionally, the tissue inhibitors of metalloproteinase enzymes (TIMP) are produced in the synovium and inhibit the destructive effects of MMPs. Excess TIMP relative to the level of MMP has been proposed as a mechanism to regulate the activity of MMPs in synovial tissue [75].

**Macrophages**

The synovial infiltrates are rich in monocytes that mature into resident macrophages. Upon activation, monocytes/macrophages directly interact with other synovial cells including synovial fibroblasts (Figure 5). Such cell interaction is a crucial event in the inflammation and tissue damage in RA, since it elicits the induction of cytokines like IL-1, IL-6, TNF-α and GM-CSF, as well as of chemokines such as IL-8 and RANTES [76]. Macrophages express numerous cell-surface receptors including CD14. Neutralization of CD14 using monoclonal antibodies has been shown to reduce production of pro-inflammatory IL-6 induced by *in vitro* interactions of monocytes and synoviocytes [77]. Positive correlation of local disease activity with the number of macrophages in the synovial sublining further highlighted a possible role for synovial tissue macrophages as biomarkers [78]. Compared to other synovial cells, macrophages have the advantage to accumulate early in the pathogenesis of RA. Further, changes in the number of synovial sublining macrophages could be used to predict efficacy of anti-rheumatic treatment, again supporting the use of synovial sublining macrophages as a tool for assessment of novel therapeutics [78, 79].

**B cells**

The inflamed RA synovium consists of numerous B cells, organized into large nodular aggregates. B cells express the maturation marker CD20 and proliferation antigens such as Ki67. The primary function of a B cell is the production of antibodies. While in a normal physiological state this effect is desired, in autoimmune diseases like RA the formation of autoantibodies leads to the destruction of tissue and the perpetuation of inflammation.
Autoantibodies recognize self antigens from host tissue and form immune complexes. The antigen-antibody structure subsequently activates either the complement cascade or phagocytosis via Fc receptors and thus destroys the patient's own tissue. Specifically, rheumatoid factors and anti-citrullinated protein antibodies have been considered as the representative autoantibodies in RA [80]. RFs recognize the Fc region of immunoglobulin G (IgG). Immune complexes containing RFs are thought to be deposited in the rheumatoid synovium which may activate inflammatory processes via activation of the complement cascade. Whereas RFs occur not exclusively in patients with RA but are also detected in other autoimmune diseases such as Sjögren's syndrome and in chronic infections, autoantibodies to citrullinated proteins show a remarkable specificity for RA [81]. Besides autoantibody production, B cells are efficient antigen presenting cells for T cells, producers of proinflammatory cytokines and modulators of leukocyte infiltration and angiogenesis [80].

Proof for a B cell involvement in RA came in 2001, when application of an anti-CD20 antibody, termed Rituximab, clearly showed efficacy in five rheumatoid patients refractory to multiple DMARDs [82]. However, the precise role of B cells in the rheumatoid synovium is still unclear. Since plasma cells do not express CD20 and are therefore not targeted by Rituximab, B cell depletion only modestly reduces autoantibody formation, suggesting a complex mechanism of action.

**T cells**

In the RA synovium, T cells constitute about 30 to 50 % of cells, most of which CD4+ with Th1 phenotype. Thus, RA was historically considered a Th1 disease. Whereas activated Th1 cells stimulate a B cell response, CD8+ T cells directly kill pathogens by production of cytotoxins such as perforin and granzymes [83].

T cells are implicated in the disease pathogenesis by the genetic association of RA with risk factors involved in T cell regulation including PTPN22, CTLA4 and class II MHC alleles [53]. Autoreactive T cells transferred to arthritis-susceptible rat strains were observed to be efficient to transfer the RA-like disease [84]. Identification of a new T cell subset, the IL-17 producing Th17 cells, shed new light on the influence of T cells in the pathogenesis of RA. Levels of IL-17 were observed to be elevated in arthritic joints and overexpression of IL-17 in the murine knee joint induced inflammation and bone erosion [85]. However, the role of T cells in RA is challenged. First, T cell derived cytokines are rarely found in the arthritic joint. Second, elimination of T cells has proven ineffective in the treatment of RA so far [86]. Despite this fact, it has now been recognized that inhibition of the
activation of T cells might be a crucial step since inhibitory antibodies targeting the T cell co-stimulatory molecule CTLA4 proved clinically effective [87].

**Figure 5: Cytokine network in rheumatoid arthritis.**
The pathogenesis of RA consists of a complex network of cytokine and cell-cell interactions. Synovial macrophages and fibroblasts produce pro- (+) and anti-inflammatory (-) proteins, that can activate either themselves or their neighbouring cells. Modified from Firestein, Nature, 2003.

**Cytokines**

In the pathogenesis of RA an interdependent network of cytokines plays a central role in mediating inflammation and tissue destruction (Figure 5). Cytokines constitute a group of small proteins capable of conveying information between and within cells. They signal via binding to specific cell surface receptors to elicit a biological response in an autocrine, paracrine or juxtacrine dependent manner. A large number of cytokines are known to be active in the arthritic joint and contribute to the pathophysiological processes underlying inflammation, articular destruction and the comorbidities associated with RA [88].

**Interleukin-1**

Interleukin-1 (IL-1) is a key mediator of synovial inflammation since it stimulates the production of pro-inflammatory mediators such as IL-6, IL-8 and prostaglandin E₂ upon binding to its cognate receptor IL-1RI. Furthermore, IL-1 increases the expression of adhesion molecules on endothelial venules, allowing the immigration of inflammatory cells into the joint space. IL-1 has also been shown to be pivotal in destructive processes, as it activates the production of MMPs and blocks cartilage repair via inhibition of collagen and proteoglycan synthesis [89].
Synovial macrophages are the main source of IL-1 in the joint [90]. The production of IL-1 is induced upon various stimuli, such as cell-cell interactions, denatured proteins, hormones and cytokines themselves. Its biological activity can be antagonized in at least four different ways. First, natural autoantibodies specific for IL-1 neutralize the activity of the cytokine. Second and third, both the soluble receptors as well as the decoy receptors lacking the cytoplasmic signalling domain are not capable of signal transduction. Fourth and most importantly, naturally occurring IL-1 receptor antagonist (IL-1Ra) binds to the IL-1RI with high affinity, thus blocking the access of IL-1 to its receptor [89]. The biological significance of IL-1Ra has been shown in knock-out mice that showed a significantly earlier onset of collagen-induced arthritis, accompanied by increased disease severity [91]. In line, mice overexpressing IL-1Ra showed a significant reduction in both incidence and severity of collagen-induced arthritis (CIA). All these data confirm the crucial role of IL-1 in the pathogenesis of CIA. However, therapy of RA patients with a recombinant IL-1Ra, Anakinra, displayed limited success. About 20 percent of patients treated with Anakinra achieved an ACR50 response, meaning that only one fifth of the patients achieved a 50 percent improvement in their disease activity scores [92].

**Interleukin-6**

The various names used initially for IL-6 reflect the pleiotropic effects of this cytokine. Originally identified as B-cell stimulatory factor-2, hence reflecting its B cell stimulating properties, IL-6 is nowadays known to be involved in diverse cellular processes such as the differentiation of T lymphocytes and the regulation of acute phase response proteins like CRP by the liver [93]. IL-6 is a secreted 26 kDa cytokine that can be produced by a wide variety of cells. In the rheumatic joint, synovial fibroblasts are the primary source of IL-6. Cultured RASF constitutively express this cytokine but its production is markedly increased upon IL-1 or TNF-Üstimulation [42]. IL-6 induces cell signalling not only via the classic pathway by binding the membrane-bound receptor IL-6RÜbut also via transsignalling when IL-6 binds the soluble form of its receptor (IL-6RÜk). Since IL-6RÜ is expressed only by hepatocytes, phagocytes and lymphocytes, the soluble form enables cells not expressing the receptor to respond to IL-6. Once IL-6 is bound by its receptor, the complex binds to ubiquitously expressed gp130. Dimerization of these three molecules activates the Jak1/STAT3 signalling pathway, inducing the expression of genes promoting inflammation and lymphocyte migration [94].
High concentrations of IL-6 are found in RA synovial fluid and plasma. Similarly, IL-6 overexpression has been found in several mouse models of autoimmunity [94]. When IL-6 was knocked out in mice, inflammatory insults induced only little changes in serum levels of acute phase proteins, thus highlighting IL-6 as a key player in inflammation [95]. In addition, IL-6 affects quality of bone and cartilage, since it decreases the production of type II collagen and aggrecans by chondrocytes. Moreover, IL-6 increases cell infiltration due to induction of chemokines by endothelial cells. All these events are crucial in the pathogenesis of RA, since they result in the development of the so-called rheumatoid “pannus” [94].

Further proof of the importance of IL-6 in the disease progress was shown upon IL-6 targeting treatment strategies. Initially, monoclonal antibodies neutralizing IL-6 were developed. Since the antibodies formed immune complexes and thus increased the half-life of the cytokine, this approach failed efficacy. Another strategy involved neutralization of the receptor instead of IL-6 itself. Indeed, a humanized monoclonal antibody to IL-6R, termed Tocilizumab, proved to be effective in blocking both the classical and transsignalling pathways of IL-6. Since 2009, Tocilizumab is licensed in Europe and used in daily clinical practice to treat RA [96].

Tumor Necrosis Factor
In 1975, TNF-α was originally identified as a factor inducing necrosis of tumours [97]. Since then, numerous reports have demonstrated additional effects of this 17 kDa cytokine, including its ability to stimulate chondrocytes to degrade animal cartilage in vitro [98], its chemotactic properties for neutrophils and monocytes in vivo [99] and its capability to promote inflammation via induction of cytokines and adhesion molecules [100, 101]. TNF-α is nowadays recognized as a cytokine playing a pivotal role in RA. The soluble molecule is primarily synthesized by synovial macrophages in RA tissues, as it was shown by in situ hybridization techniques and immunostainings [102].

Using the collagen-induced arthritis mouse model of RA, it has been shown that TNF-α neutralizing antibodies reduced both joint inflammation and damage [103]. Furthermore, TNF-α transgenic mice spontaneously developed a polyarthritis characterised by cartilage destruction, bone erosion, leukocyte infiltration and joint inflammation – all hallmarks of rheumatoid arthritis [22]. On single cell level, TNF-α stimulates the production of collagenases, PGE2, cytokines and MMPs in RASF [104]. In line, levels of cytokines were back to normal upon blockade of TNF-α.
TNF-Â mediates its effects by binding to one of the TNF receptors p55 or p57. While monocytes, macrophages and natural killer cells display both receptors on their plasmamembranes, fibroblasts and epithelial cells predominantly express p55 [105]. Interestingly, in synovial cells from patients with RA, both receptors were found to be increased [106].

The importance of TNF-Â in RA has been demonstrated in a series of clinical studies, when therapeutic neutralization of TNF-Â reached efficacy in the majority of patients. Thereby both the inhibitory TNF-Â monoclonal antibodies, Infliximab and Adalimumab, and the neutralizing soluble TNF receptor Etanercept achieved significant clinical improvements [107-109]. In fact, all of these TNF-Â blockers were shown to profoundly inhibit erosion of bone [110-112], thus linking inflammation with bone damage via TNF-Â. However, such exciting data on TNF inhibitors in RA is still challenged by the finding that at least one-third of patients produce an inadequate response to these drugs [113].

Chemokines

Cell infiltration into the synovial tissue is a hallmark of RA. Chemoattractant cytokines termed chemokines contribute to the development of a Â“pannusÂ” through guiding cells to the inflammatory site of the synovium. Activated RASF produce various chemokines such as IL-8, CCL2 and CCL5. IL-8 is considered as one of the crucial chemokines in RA, since it has been detected in synovial fluids and tissues as well as in sera of RA patients. Intriguingly, intraarticular injection of IL-8 induced synovial inflammation in rabbit knee joints whereas administration of neutralizing antibodies to IL-8 prevented arthritis [114].

Apoptosis

The synovium in RA is marked by intimal lining hyperplasia, most likely because of an imbalance between cell proliferation, survival, and death [42]. Although the synovium in RA represents a toxic environment with abundant reactive oxygen and nitrogen species, and plentiful DNA strand breaks in the lining are seen, only a few apoptotic nuclei have been detected in the synovial tissue [115, 116]. Moreover, electron microscopic studies showed that completed apoptosis is extremely rare within the intimal lining [115]. Thus, the finding of impaired cell death resulted in an effort to study both the intrinsic and extrinsic apoptotic pathways in RA.

Cell death via apoptosis results in the activation of caspases, subsequently leading to the cleavage of key cellular proteins such as cytoskeleton and DNA [117, 118]. The resulting
cellular debris are removed by phagocytes [119]. To date, three main routes to caspase activation are known: the extrinsic, the intrinsic and the granzyme B dependent pathway of apoptosis. All pathways lead to the activation of the major effector caspases, namely caspase-3, caspase-6 and caspase-7 [119].

**Extrinsic apoptosis pathway**

The extrinsic pathway is triggered from outside the cell with the binding of an apoptotic signal, such as FasL (Fas ligand, CD95L) and TRAIL (TNF related apoptosis inducing ligand), to one of the six transmembrane death receptors (DR): Fas, TNFR1, DR3, DR4 and DR5 (also called TRAIL-R1 and TRAIL-R2), and DR6 [120]. Once activated, the death-inducing signalling complex (DISC) is formed, consisting of caspase-8 and of the two adapter proteins FADD and TRADD. The caspase gets activated upon its cleavage, and apoptosis is promoted by the activation of downstream effector caspases, such as caspase-3 and -7.

**Intrinsic apoptosis pathway**

In the intrinsic pathway, apoptosis is mediated from within the cells, usually in response to cellular signals resulting from cellular stresses such as DNA damage, cell cycle dysregulation, hypoxia, or loss of cell survival factors. Cleavage of members of the BH3-only protein family overcomes the inhibitory effect of the anti BCL-2 family members and cytochrome c gets released from the mitochondria into the cytosol. The resulting formation of an apoptosome leads to the activation of caspase-9, propagating a proteolytic cascade of effector caspase activation events [119, 121, 122].

**Granzyme B dependent apoptosis pathway**

Beside the extrinsic and intrinsic pathways of apoptosis, granzyme B has been shown to be an inducer of apoptotic cell death [123]. The protease is delivered into target cells by cytotoxic T lymphocytes and natural killer cells. Once successfully entered the cell, granzyme B is able to directly activate caspases to initiate apoptosis [119].
Increased cell numbers accumulate in the synovium as a result of insufficient cell deletion. Synovial cells from patients with RA show decreased susceptibility to spontaneous as well as FasL and TRAIL mediated apoptosis [124, 125]. Although Fas is constitutively expressed by RASF, apoptosis is initiated only in a few cells when engaged with FasL [126]. The ubiquitin-like protein Sentrin-1 was suggested to protect cells from Fas induced apoptosis by the modification of apoptosis related proteins such as p53 and other molecules involved in the JNK pathway [127]. Even though pro-apoptotic p53 is present in high concentrations in the rheumatoid lining, it seems to rather direct synoviocytes to cell cycle arrest through
expression of p21 instead of inducing apoptosis through PUMA (p53 upregulated modulator of apoptosis) [42]. In line, PUMA was found to be scarcely expressed in RA synovial cells but sufficiently induced cell death upon artificial overexpression [128, 129]. Furthermore, somatic mutations were detected in the p53 gene, leading to functional inactive p53 [66]. The beneficial effects of wildtype p53 were confirmed in a rabbit model of arthritis, when intraarticular injection of p53 induced synovial apoptosis and reduced inflammation of the arthritic joint [130]. Some years later, the experiment was repeated with adenoviral transfer of TRAIL instead of p53 overexpression and again, a decrease in synovial inflammation was observed [131]. TRAIL was reported to act via inhibition of the PI3K/Akt molecular pathway [132]. Similar, the tumour suppressor PTEN (phosphatase and tensine homolog on chromosome ten) has been shown to act via antagonism of PI3K/Akt signalling but was observed to be rarely expressed in the RA synovium [128, 132], suggesting a possible role of TRAIL in the deregulation of apoptotic pathways in RA. Taken together, synovial lining hyperplasia in RA appears to result from an imbalance between the expressions of various pro- and anti-apoptotic stimuli during the course of inflammation (Figure 6).

**Epigenetic regulations**

Regulation of gene activity is a key step for every single cell, since proper cell function requires precise expression or repression of particular sets of genes. In contrast to genetics, epigenetic changes are heritable changes in gene function that do not alter the sequence of the DNA. The epigenome acts as an interface between the genome and the environment, that should be considered at two levels: the cellular environment and the environment surrounding an entire organism [133].

The two major types of epigenetic regulation mechanisms are methylation of DNA and post-translational modifications of histones [133]. In addition, alterations in the expression of microRNAs (miRNA, miR) are another mechanism of posttranscriptional gene regulation. The aggressive phenotype of synovial fibroblasts from individuals with RA is thought to be an intrinsic property. Indeed, RASF show epigenetic abnormalities, represented by DNA hypomethylation, histone hyperacetylation and miRNA expression alteration [134].

**DNA methylation**

DNA methylation refers to the covalent attachment of a methyl group (CH₃) to cytosine residues. Most frequently, cytosines found in CpG dinucleotide sequences are the targets of
DNA methylation. CpG dinucleotides are underrepresented in the genome and enriched in regions known as CpG islands. Methylation of DNA is generally associated with transcriptional repression, especially when the methylated sites involve promoters or other gene regulatory regions. However, DNA methylation may be activating if it prevents binding or limits expression of transcriptional repressors [135]. Methylation requires the activity of a group of enzymes known as DNA methyltransferases (DNMTs) to establish and maintain CpG methylation. DNMTs use S-adenosylmethionine as methyl group donor. Inhibition of DNMTs results in passive DNA demethylation [133].

A recent study provided evidence that the DNA isolated from RASF is hypomethylated and additionally, that RASF have lower levels of 5-methylcytosine than SF from OA patients [136]. Furthermore, treating normal SF with the DNA methyltransferase inhibitor 5-azacytidine mimicked the aggressive phenotype seen in RASF [136], suggesting that alterations in DNA methylation contribute to the pathogenesis of RA.

Histone modifications

DNA is highly organized within the chromatin and tightly wrapped around histones. A highly compact state of chromatin, referred to as heterochromatin, reduces the accessibility of the DNA thus decreasing the general transcriptional rate of gene expression. In contrary, euchromatin comprises DNA in an open state and therefore accessible for polymerases and transcription factors to initiate transcription [133]. Switching between these two states is dependent on the presence of specific histone tags. Histones carry flexible N-terminal tails that are accessible for various post-translational modifications, including phosphorylation of serines or threonines, acetylation of lysines, methylation of lysines or arginines, and ubiquitination [135]. Of these, acetylation and methylation are the most intensively studied. Whereas histone acetylation by histone acetyltransferases (HATs) renders the chromatin in an active state, histone deacetylation by histone deacetylases (HDACs) generally results in silencing of affected genes [137]. Upon methylation, genes are either activated or repressed, depending on the specific residue of histones modified [138]. As with DNA methylation and histone acetylation, also histone methylation is an enzymatically reversible modification.

Histone deacetylases

Despite their names, HATs and HDACs also act on proteins other than histones. Nowadays, they are also called lysine deacetylases (KDAC), as they target not only histones [139]. There are four different groups of HDACs, based on function and DNA sequence similarity. The
human class I includes HDAC 1, 2, 3 and 8, class II consists of HDAC 4-7, 9 and 10. The activities of these enzymes are inhibited by trichostatin A (TSA) and thus, the members of these two groups are considered classical HDACs. The third group of HDACs consists of the NAD+-dependent sirtuins, whose activities are not affected by TSA. Last, HDAC 11 belongs to the class IV and is, like the classical HDACs, dependent on zinc [140].

During deacetylation, an acetyl group (CH₃CO) is removed from the lysine residues in the N-terminal tail of proteins. The conversion of acetyl-lysine to the positively charged lysine alters the protein structure and thus the interactions of the modified protein with other molecules. Beside directly influencing the protein activity, acetylation might also crosstalk with other post-translational tags such as phosphorylation and methylation to dynamically control cellular signalling [141].

Inhibitors of HDACs (HDACi) have shown therapeutic utility in the treatment of different inflammatory disorders such as colitis, airway inflammation, asthma and rheumatoid arthritis [142-144]. Regarding RA, studies using the HDACi TSA demonstrated successful application in an arthritis mouse model, where TSA reduced the clinical scores of arthritis and synovial inflammation [145, 146]. Similar results were obtained using the HDACi FK-288 in a mouse model of autoantibody-mediated arthritis. Intravenous application of FK-228 rapidly reduced the symptoms of arthritis, accompanied by a marked decrease in the expression of TNF-\(\text{\textalpha}\) and IL-1\(\beta\) in the synovium. FK-288 was found to mediate its effect through induction of the cyclin-dependent kinase inhibitors p16INK4a and p21 [147]. However, the beneficial effects of HDACi are challenged by the finding, that the balance of HAT/HDAC activity is strongly shifted towards histone acetylation in RA synovial tissues [148]. Further analysis is needed to rule out whether inhibition of HDACs or rather rebalance of a proper HAT/HDAC activity level is feasible for the treatment of RA.

Sirtuins (silent mating type information regulation 2 homolog)

Recently, sirtuins emerged as important regulators of a variety of important biological processes. In mammals, seven sirtuins SIRT1-7 have been described with quite diverse substrate specificity, cellular functions and subcellular distributions [149].

SIRT1 and 6 are found in the nucleus, SIRT2 is located in the cytoplasm, SIRT3-5 in the mitochondria and SIRT7 in the nucleolus. However, recent data suggest trafficking of individual SIRTs between different subcellular compartments. SIRT1 carries two nuclear localization signals and two nuclear export signals. It has been shown that the nucleocytoplasmic transfer is a regulatory mechanisms of the activity of SIRT1 [150].
Similarly, SIRT2 was found to shuttle between the cytoplasm and the nucleus [151]. Several acetylated substrates have been identified for all the sirtuins except SIRT4. However, SIRT4 as well as SIRT6 exert ADP-ribosyltransferase activity [149]. Although each SIRT is different, they all share a common characteristic as they require nicotinamide adenine dinucleotide (NAD+) as an essential cofactor [149].

Being dependent on NAD+, the activity of SIRTs is closely linked to the metabolic state of cells and thus, changes in the cellular NAD+ have a significant impact on physiological processes. Biosynthesis of NAD+ is accomplished through either the *de novo* pathway from tryptophan or the salvage pathway from preformed compounds containing nicotinamide [152]. Nampt, also known as PBEF or visfatin, catalyzes the conversion from nicotinamide into nicotinamide mononucleotide, the rate-limiting step in NAD+ biosynthesis in the salvage pathway. Only recently, Nampt was identified as a marker of inflammation in RA [153]. Levels of Nampt in serum and synovial fluid positively correlated with the degree of inflammation and clinical disease activity. Being overexpressed in synovial tissues from RA patients and known to regulate intracellular NAD+ levels and thus NAD+-dependent enzymes, Nampt might highly influence the activity of sirtuins.

Taken together, the dependence of SIRTs on disregulated Nampt implies aberrant functions of the sirtuins in the pathogenesis of RA as well. Based on this, we performed an expression profile of sirtuins in synovial fibroblasts from patients with RA and non-inflammatory OA (Figure 7). Indeed, aberrant high mRNA levels of sirtuins were observed in RASF when compared to OASF, thus supporting the hypothesis of a crucial involvement of SIRTs in RA. Being best characterized so far and among the most abundantly expressed sirtuins, the function of SIRT1 in inflammation and apoptosis in RA was further analyzed.
Figure 7: Expression of SIRTs mRNA in synovial fibroblasts from patients with RA and OA.
RT-PCR showing relative expression of SIRTs mRNA in synovial fibroblasts from patients with RA and OA. Results are presented as the difference in threshold cycle [delta Ct], relative to the endogenous control 18S rRNA. * = p<0.05, unpaired t-test.

Sirtuin 1

The role of SIRTs in ageing was discovered in budding yeast, where overexpression of the SIRT1 homolog, Sir2, increased replicative lifespan [154]. Furthermore, SIRT1 was suggested as the mediator of the beneficial effects of calorie restriction (CR). CR prolongs lifespan in mice and probably humans, possibly via enhancing SIRT1 activity. Thus, increasing levels of SIRT1 are thought to diminish the symptoms of an unhealthy high caloric diet.

Supporting this idea, SIRT1 was proposed as the explanation of the French paradox: Certain populations, including the French and the Greek, suffer little heart disease despite high intake of saturated fat. It has been suggested that regular wine consumption reduced the risk of coronary heart disease [155]. The grape and wine compound resveratrol was identified as the mediator of the beneficial effects of moderate wine consumption and to extend lifespan [156]. Excitingly, resveratrol is known to be a SIRT1 activator [157]. In a screening assay, resveratrol was identified as a compound boosting the deacetylation of synthetic substrates labelled with a chemical fluorophore group [157]. However, recent studies challenged the SIRT1 dependent mode of action of resveratrol [154, 158, 159]. No protein activation could be detected upon addition of resveratrol when nonfluorophore-containing peptides instead of fluorophore-labelled substrates were used, suggesting that resveratrol interacts rather with the fluorophore than with SIRT1.
Introduction

As with resveratrol, existing data on the effect of the pan sirtuin inhibitor, sirtinol, is controversial. Sirtinol is a selective inhibitor of the enzymatic activity of SIRTs without affecting classical HDACs [160]. Treatment of human dermal cells with sirtinol reduced inflammatory responses to TNF-\(\tilde{\text{U}}\) or IL-1\(\tilde{\text{B}}\) stimulated cells through down regulation of adhesion molecules and of chemotactic CXCL10 and CCL2 [161]. Although the mode of action and the physiological role of SIRT modulating compounds remain unknown, SIRT1 is emerging as a master regulator of metabolic and stress responses with several critical downstream targets. SIRT1 has been shown to regulate transcription factors important in energy metabolism, such as PGC-1\(\tilde{\text{U}}\) and members of the FOXO family [162, 163]. Moreover, SIRT1 was found to physically interact with NF-\(\tilde{\text{EB}}\), a crucial transcription factor involved in the regulation of inflammatory responses [164]. Upon deacetylation of the RelA/p65 subunit, NF-\(\tilde{\text{EB}}\) was inhibited to interact with promoter regions of target genes to enhance transcription. Similarly, macrophages from myeloid SIRT1 knock out mice displayed increased NF-\(\tilde{\text{EB}}\) mediated inflammation in response to environmental stress, indicating an anti-inflammatory activity of SIRT1 [165]. However, published data on the effects of SIRT1 on inflammation is controversial. Inhibition of SIRT1 was shown to attenuate antigen-induced airway inflammation and hyperresponsiveness [166]. In agreement, a sirtuin activator had an inhibitory effect on LPS induced inflammation in intraperitoneal murine macrophages [167].

Figure 8: Structural model of SIRT1 complexed with its enzymatic inhibitor EX-527.

The SIRT1 specific inhibitor EX-527 docks at its target to inhibit SIRT1\(\tilde{\text{E}}\) activity. The surface of SIRT1 is coloured brown for lipophilic and blue for the hydrophilic areas. Atoms of EX-527 highlighted in blue have hydrogen bond donors binding to the polar surface of SIRT1. From Huhtiniemi, J Comput Aided Mol Des, 2006.
Furthermore, SIRT1 is also included in the regulation of DNA repair mechanisms via regulation of Ku70 [168] and PARP1, the circadian clock modulation via BMAL1 and PER2 [169, 170] and general stress responses via targeting HIF-1α, HSF-1 and p53 [171]. Of note, through the suppression of p53 functions, SIRT1 acts as an oncogene. Increased levels of SIRT1 are found in several human tumours and are followed by a decrease in the active p53, leading to genome instability and resistance to apoptosis [172, 173]. Similarly, deacetylation of the DNA repair factor Ku70 by SIRT1 leads to inhibition of apoptotic pathways. Contrary to these findings, in HEK293 epithelial cells, SIRT1 augmented apoptosis in response to TNF-α [164], highlighting that SIRT1 might have different biological outcome depending on the apoptotic stimuli and on the different cell types. The breadth of SIRT1 targets highlights the importance of a well balanced expression and activity state of this protein within a cell (Figure 8).

MicroRNA regulation
The family of small non-coding microRNAs has emerged as a new modulator of gene expression. MiRNAs have been recognised as potent regulators of important biological processes such as proliferation, apoptosis and cellular differentiation [174]. The approximately 22 nucleotides long miRNAs can influence mRNA processing at the posttranscriptional level by degradation of target mRNA or by inhibition of protein translation. Human miRNA genes are often clustered and are present within exons and introns.

During their biogenesis, miR genes are transcribed by RNA polymerase II into primary miRNAs (pri-miRs) containing hairpin structures. In the nucleus, the pri-miR is cleaved by the ribonuclease Drosha to generate a precursor miRNA (pre-miR) hairpin. Pre-miRs are then transported into the cytoplasm by exportin 5 and are processed by Dicer to generate a miR duplex consisting of a mature and a passenger miR (also called miR star, miR*) strand [175]. The miRNA is then loaded onto an argonaute protein to form the RISC complex, where binding to the cognate mRNA sequence and translational repression and/or degradation takes place (Figure 9). Whereas it was previously thought that exclusively mature miR strands exert biological effects, there is nowadays increasing evidence that also miR* strands are functional and inhibit the biosynthesis of proteins [176, 177].
Processing of miRs includes the production of the primary miRNA transcripts (pri-miRNAs) and subsequent cleavage by Drosha within the nucleus. The resulting precursor hairpin structure, the pre-miRNA, is exported from the nucleus by exportin 5. In the cytoplasm, the RNase Dicer cleaves the pre-miRNA hairpin into its mature length. Either the mature or the passenger miR strand enters the RISC complex, resulting in degradation or translational stop of the target mRNA. Modified from Ceribelli et al., FEBS, 2011.
MicroRNA-34a

MiR-34a has been shown to modulate apoptotic pathways in different cell types. However, existing data are controversial. On the one hand, miR-34a was shown to be an inducer of programmed cell death in colon cancer cell lines and mouse embryonic fibroblasts [178-180]. On the other hand, miR-34a protected cells, such as human B lymphocytes and a mouse tubular cell line, from apoptosis [181, 182]. It has been suggested that there is a negative feedback loop regulating the expression of SIRT1 and miR-34a via p53 [183]. Indeed, the transcription factor p53 turned out to be a direct regulator of miR-34a. In this work, we confirmed reports showing modulation of SIRT1 expression upon miR-34a (Figure 10).

Although an initial screening suggested down regulation of miR-34a in SF from patients with RA compared to OA, individual Real-time PCR analysis could not confirm this finding. Excitingly, the expression of the passenger strand miR-34a* was found to be highly down regulated in RASF. Like the mature miR-34a strand, miR-34a* is generated upon cleavage of the pre-miR-34a/34a* hairpin structure. Although the sequences are highly complementary, a few nucleotides differ between both strands (Figure 11). So far, no existing data on the effects of miR-34a* has been published.

![Figure 10: MiR-34a modulates SIRT1 expression.](image)

Levels of SIRT1 mRNA after overexpression (pre-34a, n=8) or silencing (anti-34a, n=3) of miR-34a are shown. Results are the difference in comparative threshold cycle (delta Ct) between SIRT1 and the internal control, 18S rRNA and presented relative to control transfected cells (x-fold).
Figure 11: Hairpin structure of pre-miR-34a/34a*.
The pre-miR-34a/34a* hairpin structure consists of the mature miR-34a (depicted in purple) and the passenger miR-34a* (depicted in red) strand. Differences in complementary nucleotides between miR-34a and miR-34a* sequences are highlighted in green.
AIM OF THE STUDY

Insufficient apoptosis of synovial cells is a key mechanism contributing to the characteristic hyperplasia of the synovial tissue in the arthritic joint [184]. Prolonged longevity of RA synovial fibroblasts goes along with increased levels of joint inflammation and destruction of bone and cartilage. Thus, synovial fibroblasts play a key role in the pathogenesis of RA since there is evidence that RASF are intrinsically activated to become aggressive and destructive cells [185]. First, SF from patients with RA produce large amounts of proinflammatory cytokines. Second, they actively drive joint degradation through production of matrix destructive enzymes. And third, RASF display an apoptosis resistant phenotype that potentiates their inflammatory and destructive effects [186]. Elucidation of the underlying mechanisms resulting in apoptosis resistance might markedly improve the general understanding of RA pathogenesis and thus, might open new possibilities to optimize the treatment of RA.

Recently, the NAD+-dependent class III of histone deacetylases, sirtuins, emerged as key regulator of diverse biological processes, such as apoptosis. In particular, SIRT1 has been shown to modulate apoptotic pathways in different cell types and furthermore, to be involved in inflammatory processes. In addition, there is evidence for deregulated levels of NAD+ in the RA synovium. Nampt, also called PBEF, catalyses the rate-limiting step in the biosynthesis of NAD+ and has been observed to be constitutively over expressed in RA synovial cells. By regulating intracellular NAD+ levels, Nampt influences the activity of the NAD+ dependent sirtuins. So far, SIRT1 expression and function in the RA synovium has not been analyzed. We hypothesized that SIRT1 activity and/or expression might be altered in RA and thus, contributes to the apoptosis resistance of RA synovial cells and might also influence inflammatory pathways.

To address these questions, we established in Chapter II a quantitative expression profile of SIRT1 in synovial cells from patients with RA and compared the relative expression levels to OASF or healthy control cells. Using stimulation experiments, the regulation of SIRT1 in RA synovial cells was investigated in vitro. Since we found high levels of SIRT1 in RA and the production of SIRT1 to be further induced upon stimulation with TNF-Î”, we analyzed the effect of SIRT1 on apoptosis and on production of inflammatory mediators in synovial fibroblasts and monocytes.
The small non-coding microRNAs emerged as crucial modulators of a variety of important biological pathways. In RA synovial cells, the altered expression pattern of miRs was found to increase production of pro-inflammatory cytokines and destructive MMPs, thus driving joint destruction and inflammation in RA.

MiR-34a has already been shown to modulate apoptotic pathways via targeting SIRT1. Since a preliminary screening assay on 240 miRs suggested down regulation of miR-34a in RASF, we assumed involvement of miR-34a in the sirtuin dependent apoptosis pathway of RA synovial cells. However, we could not confirm deregulated expression of miR-34a in RA, but excitingly found the passenger strand of miR-34a to be highly reduced in synovial cells from RA patients. In Chapter III, we elucidated the functional impact of down regulated miR-34a* in the pathogenesis of RA. We observed the low levels of miR-34a* to contribute to the apoptosis resistance in synovial fibroblasts from patients with RA. Furthermore, we investigated the molecular pathways by which miR-34a* modulates apoptotic cell death.

In summary, we examined in this work whether epigenetic regulation of gene expression might influence the phenotype of RA synovial cells. We aimed to elucidate the functional consequences of high SIRT1 and low miR-34a* levels in RA and investigated their role on cellular longevity and inflammation.
CHAPTER II  SIRT1 overexpression in the Rheumatoid Arthritis synovium contributes to proinflammatory cytokine production and apoptosis resistance


Fabienne Niederer, Caroline Ospelt, Fabia Brentano, Michael O. Hottiger, Renate E. Gay, Steffen Gay, Michael Detmar, Diego Kyburz
ABSTRACT

Objective:
The analysis of the expression of SIRT1 in synovial tissues and cells of rheumatoid arthritis (RA) patients and to study the function of SIRT1 in inflammation and apoptosis in RA.

Methods:
Levels of SIRT1 expression were analyzed in synovial tissues and cells from RA patients by Real-time PCR and Western blotting before and after stimulation with Toll-like receptor (TLR) ligands, tumor-necrosis factor alpha (TNF-α) and interleukin (IL)-1β. Immunohistochemistry was used to study the localization of SIRT1. FACS analysis was performed to investigate the effect of SIRT1 on apoptosis. Peripheral blood monocytes and RA synovial fibroblasts (RASF) were transfected with wild type or enzymatically inactive SIRT1 expression vectors or with siRNA targeting SIRT1. Cytokine analysis of IL-6, IL-8 and TNF-α were performed by ELISA to study the role of SIRT1 on proinflammatory mediators of RA.

Results:
SIRT1 was found to be constitutively upregulated in synovial tissues and cells from RA compared to osteoarthritis (OA) patients. TNF-α stimulation of RASFs and monocytes resulted in further induced expression levels of SIRT1. Silencing of SIRT1 promoted apoptosis in RASFs, whereas SIRT1 overexpression protected cells from apoptosis. Inhibition of SIRT1 enzymatic activity by inhibitors, siRNA and overexpression of an enzymatically inactive form of SIRT1 reduced lipopolysaccharide (LPS)-induced levels of TNF-α in monocytes. Similarly, knockdown of SIRT1 resulted in a reduction of proinflammatory IL-6 and IL-8 in RASFs.

Conclusion:
The TNF-α induced overexpression of SIRT1 in RA synovial cells contributes to chronic inflammation by promoting proinflammatory cytokine production and inhibiting apoptosis.
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by the destruction of joint cartilage and bone. Synovial hyperplasia and persistent synovial inflammation with infiltration of inflammatory immune cells into the synovial lining are hallmarks of RA [187]. Innate immunity was shown to be important for the development of chronic arthritis. Activation of Toll-like (TLR) and NOD-like receptors in synovial cells leads to the expression of several proinflammatory genes, such as interleukin-6 (IL-6) and tumor-necrosis factor alpha (TNF-α) [44, 45]. We have previously reported the induction of the adipokine PBEF, also called Visfatin, upon stimulation of TLRs [153]. PBEF/Visfatin catalyses the rate-limiting step in the biosynthesis of nicotinamide-adenine-dinucleotide (NAD+) [188]. By regulating intracellular NAD+ levels, PBEF/Visfatin influences the activity of a variety of NAD+ consuming enzymes, including the sirtuins (SIRT) [189, 190].

SIRTs are a conserved family of NAD+ dependent histone deacetylases (HDAC) and mono-ADP-ribosyltransferases that target histones, transcription factors, and coregulators to adapt gene expression to the cellular energy state [191-193]. In mammals seven sirtuin genes – SIRT1 to SIRT7 – have been identified. Among them, SIRT1 is best characterised so far and has been shown to regulate transcription factors such as p53 [172], members of the forkhead transcription factor FOXO family [162], the DNA repair factor Ku70 [168], NF-κB [164] and the transcriptional coactivator p300 [194].

In lung cancer cell lines, SIRT1 was found to physically interact with and deacetylate the RelA/p65 subunit of NF-κB, thereby inhibiting its ability to interact with promoter regions of target genes to enhance transcription [164]. Hyperacetylation of lysine 310 of RelA/p65 rendered NF-κB highly active, resulting in increased transcription of proinflammatory cytokines such as TNF-α and IL-1 in a myeloid SIRT1 knock out mouse model [165]. However, nicotinamide has been shown to reduce the production of proinflammatory cytokines as well as IL-10 in primary human macrophages, possibly via inhibiting sirtuins [144].

The last years, SIRT1 gained much attention since its expression was shown to mediate longevity [195]. SIRT1 deacetylates the DNA repair factor Ku70 leading to inhibition of apoptosis. In addition, SIRT1 protected epithelial cells from p53-mediated apoptosis [172, 173]. Contrary to these findings, in HEK293 epithelial cells, SIRT1 augmented apoptosis in response to TNF-α [164], highlighting that SIRT1 might have different biological outcome depending on the apoptotic stimuli.
So far, SIRT1 expression and function in the RA synovium has not been analysed. Therefore, in consideration of the mentioned controversial results on the influence of sirtuins on inflammation and apoptosis, we have analyzed the expression, regulation and function of SIRT1 in RA. We show overexpression of SIRT1 in synovial tissues from RA patients. In addition, SIRT1 is shown to decrease apoptosis in synovial cells and to promote proinflammatory cytokine production.
MATERIAL & METHODS

Patients and tissue preparation. Synovial tissue biopsy specimens were obtained from patients with RA and osteoarthritis (OA), after informed consent has been obtained (Department of Orthopedic Surgery, Schulthess Clinic, Zurich, Switzerland). All RA patients fulfilled the American College of Rheumatology criteria for classification of RA [10]. Synovial tissue specimens were digested and SFs were grown as previously described [196]. The patient characteristics used in this study are shown in Table 2.

Table 2: Patient characteristics used in this study*

<table>
<thead>
<tr>
<th></th>
<th>RA patients (n=39)</th>
<th>OA patients (n=10)</th>
<th>Healthy controls (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (range) years</td>
<td>62 (29-86)</td>
<td>76 (64-98) Å</td>
<td>43 (29-64) §</td>
</tr>
<tr>
<td>Sex, no. female/male</td>
<td>31/8</td>
<td>5/5</td>
<td>4/2</td>
</tr>
<tr>
<td>Disease duration, mean (range) years</td>
<td>25 (3-50)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Medications, no. taking/no. assessed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSAIDs</td>
<td>12/39</td>
<td>3/10</td>
<td>0/6</td>
</tr>
<tr>
<td>DMARDs</td>
<td>31/39</td>
<td>0/10</td>
<td>0/6</td>
</tr>
<tr>
<td>No. RF+ (&gt;20 IU)/no. assessed</td>
<td>29/29</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CRP, mean (range) mg/liter</td>
<td>14.4 (1.0-52.5)</td>
<td>6.9 (0.9-39.5)</td>
<td>NA</td>
</tr>
</tbody>
</table>

* NA = not assessed; NSAIDs = nonsteroidal antiinflammatory drugs; DMARDs = disease-modifying antirheumatic drugs; RF = rheumatoid factor; CRP = C-reactive protein.
Å Patients with osteoarthritis (OA) were significantly older than patients with rheumatoid arthritis (RA) and healthy controls.
§ Healthy controls were significantly younger than patients with OA and RA.

Preparation of monocytes from peripheral blood. Isolation of peripheral blood mononuclear cells (PBMC) from whole blood of healthy donors or patients with RA was performed using standard Ficoll density-gradient centrifugation (GE Healthcare, Otelfingen, Switzerland). Monocytes were separated by positive selection with CD14 MACS MicroBeads or, when used for stimulation or transfection experiments, by negative selection with MACS MicroBeads Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany).

Stimulation assays. RASFs and primary peripheral monocytes from healthy individuals were plated in 12- and 24-well plates (5 x 10^4 and 3-5 x 10^5 cells/well) in 500 μl supplemented DMEM and RPMI, respectively, and stimulated for the indicated time points
with the following agents: 300 ng/ml Pam3CSK4, 10 μg/ml poly(I:C) (both InvivoGen, San Diego, CA), 10 ng/ml lipopolysaccharide (LPS from *Escherichia coli* J5; List Biologicals, Campbell, CA), 10 ng/ml TNF-α, 1 ng/ml IL-1β (both R&D Systems, Abingdon, United Kingdom), 30 μM Sirtinol (Sigma, Buchs, Switzerland) or 9 μM EX-527 (Tocris Bioscience, Bristol, UK).

**Transfection experiments.** Monocytes from healthy volunteers and RASFs were transfected using AMAXA nucleofection kits VPA-1007 and VVPI-1002 (both Lonza, Cologne, Germany), respectively. For silencing experiments, 100 μM SIRT1 validated stealth siRNA or stealth control high GC siRNA (both Invitrogen, Basel, Switzerland) were used. For overexpression experiments, 1 μg of SIRT1 wild type, SIRT1 mutant (H363Y), empty pcDNA3.1(-) (mock) or no nucleic acid (untransfected) was used. pcDNA3.1-SIRT1-MYC/HIS wild type and the catalytically inactive mutant (H363Y) were described previously [197]. Transfected monocytes were further incubated at 37°C for 18-20 hours before stimulation with LPS for 8 hours, in presence or absence of 50 μM sc-514 (Sigma, Buchs, Switzerland). RASFs were transfected for 48 hours and subsequently stimulated with LPS or TNF-α for 24 hours (siRNA) or 40 hours (vectors). Successful transfection was confirmed by Real-time PCR using SIR1 mRNA specific primers.

**Real-time polymerase chain reaction (PCR).** Total RNA was isolated with the RNeasy Mini kit including treatment with RNase-free DNase (Qiagen, Hombrechtikon, Switzerland) and reverse transcribed using random hexamers and multiscribe reverse transcriptase (both Applied Biosystems, Rotkreuz, Switzerland). Non-reverse transcribed samples were used as negative controls. Quantification of SIRT1 and IL-6 mRNA was performed by TaqMan RT-PCR using the ABI Prism 7700 Sequence Detection system (Applied Biosystems). The primer sequences are shown in Table 3. The endogenous control 18S cDNA was used for correcting the results with the comparative threshold cycle (Ct) method for relative quantification as described by the manufacturer.

**Table 3: SYBR green primers used for Real-time PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIRT1 forward</td>
<td>5′GCG GGA ATC CAA AGG ATA AT-3′</td>
</tr>
<tr>
<td>SIRT1 reverse</td>
<td>5′CTG TTG CAA AGG AAC CAT GA-3′</td>
</tr>
<tr>
<td>IL-6 forward</td>
<td>5′CTC TTC AGA ACG AAT TGA CAA ACA A-3′</td>
</tr>
<tr>
<td>IL-6 reverse</td>
<td>5′GAG ATG CCG TCG AGG ATG TAC-3′</td>
</tr>
</tbody>
</table>
**Enzyme linked immunoabsorbant assay (ELISA).** Protein in cell supernatants was detected by ELISA with OptEIA® Kits (BD Pharmingen, San Diego, CA) for TNF-α, IL-6, and IL-8 according to the manufacturer’s instructions. Absorption was measured at 450 nm and data were analysed using Revelation v4.22 software (Dynex Technologies, Denkendorf, Germany).

**Immunohistochemistry.** Synovial tissues were fixed in paraformaldehyde and embedded in paraffin. Tissue sections were deparaffinized and pretreated with 10 mM citrate buffer, pH 6. After blocking endogenous peroxidase and nonspecific binding, slides were incubated with rabbit IgG (Jackson, Suffolk, UK) or rabbit-anti-human SIRT1 antibody (E104, 1:40, Abcam, Cambridge, UK) over night at 4°C. For double staining, 2 μg/ml mouse-anti-vimentin, mouse-anti-CD68 antibodies (both Dako, Glostrup, Denmark) or mouse IgG (Jackson, Suffolk, UK) were added. Sections were incubated with biotinylated goat-anti-rabbit IgG and AP-conjugated goat-anti-mouse antibodies (Jackson, Suffolk, UK) followed by incubation with HRP-conjugated streptavidin complex (ABC Kit, Vector laboratories, Peterborough, UK). SIRT1 positive cells were visualized using DAB-Nickel (Vector laboratories, Peterborough, UK). Vimentin or CD68 positive cells were visualized using Vector Red reagent. SIRT1 single stained and IgG control slides were counterstained with Eosin.

**Immunoblotting.** For protein preparation, tissues and cells were lysed in RIPA buffer, boiled and mixed with Laemmli buffer [198]. Proteins were separated on SDS-polyacrylamide gels and transferred to Protran nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany). Membranes were probed with anti-SIRT1 antibodies (E104, Abcam) and anti-α-tubulin (Sigma, Buchs, Switzerland), respectively, and detected with HRP-conjugated secondary antibodies using the ECL Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

**Fluorescence activated cell sorting (FACS).** RASFs were transfected with siRNA or vectors as described above for 48 hours. Medium was changed and 24 hours later, cells were detached with Accutase (PAA Laboratories, Pasching, Austria) and stained for AnnexinV and PI with the AnnexinV-FLUOS Staining Kit (Roche, Mannheim, Germany). Cells were subsequently analyzed on a FACSCalibur flow cytometer. Data were processed using CellQuest software (BD Biosciences, San Jose, CA).
Results

**Statistical analysis.** Unpaired and paired t-tests or one-way ANOVA followed by Dunnett's post test were used where appropriate for statistical evaluation of the data by GraphPad Prism 5.0 software. Values are presented as mean (SEM). p-values less than 0.05 were considered as significant.
RESULTS

Elevated expression of SIRT1 in RA synovial tissues.

To study the association of SIRT1 expression with chronic joint inflammation in RA, we compared SIRT1 protein levels in RA with noninflammatory OA synovial tissues. Western blot analysis revealed higher expression of SIRT1 protein in whole synovial tissue and cultured SFs from joints of patients with RA compared to OA (Figure 12A). Real-time PCR analysis showed 5.2 fold higher levels of SIRT1 mRNA in SFs from RA compared to OA patients (p<0.01). Additionally, levels of SIRT1 mRNA were 2.2 times higher in peripheral blood monocytes from RA patients compared to healthy volunteers (p<0.01; Figure 12B).

![Figure 12: High basal protein and mRNA levels of SIRT1 in tissues, synovial fibroblasts and monocytes from RA patients.](image)

(A) Western blot showing the basal expression of SIRT1 protein from patients with RA and OA in synovial tissue samples (n=4, each) or in synovial fibroblasts (n=3, each). α-tubulin served as loading control. (B) Relative expression units of SIRT1 mRNA in synovial fibroblasts from patients with RA (n=7) or OA (n=4) and in monocytes from patients with RA (n=7) or healthy controls (n=6) are shown. Results are presented as the difference in threshold cycle [delta Ct], relative to 18S rRNA. See Table 1 for patient characteristics. * = p<0.01, unpaired t-test.

Localization of SIRT1 expression in RA synovial tissue sections.

To further analyze the expression of SIRT1 in synovial tissue sections from patients with RA, we performed immunohistochemistry. Pronounced expression of nuclear SIRT1 protein was found in the lining and sublining layers, but also in perivascular areas (Figure 13A). Double staining with SIRT1 and vimentin or CD68 showed the expression of SIRT1 in vimentin positive SFs as well as in CD68 positive monocytes/macrophages (Figures 13B and C).
**Results**

**Figure 13: SIRT1 expression in synovial tissues from RA patients.**
(A) Representative sections of RA synovial tissue specimens stained for SIRT1 or IgG control. Positive staining of SIRT1 appears as dark gray. Sections were counterstained with Eosin. (B) Representative sections of RA synovial tissue specimens double-labeled for SIRT1 and vimentin or IgG control. SIRT1 appears as dark gray, and vimentin as red. (C) Representative sections of RA synovial tissue specimens double-labeled for SIRT1 and CD68 or IgG control. SIRT1 appears as dark gray, and CD68 as red. One representative section is shown (n=4). Original magnification x400.

**SIRT1 expression is induced upon stimulation with TNF-\( \alpha \)**

To analyze the influence of activated TLR pathways and IL-1\( \beta \) on SIRT1 expression, RASFs were treated with TLR ligands and IL-1\( \beta \) for 24 hours. Real-time PCR analysis revealed no changes in the levels of SIRT1 mRNA. However, stimulation of RASFs with TNF-\( \alpha \)-revealed a modest induction of SIRT1 mRNA (Figure 15A). Time course analysis showed increased levels of SIRT1 mRNA already after 1 and 4 hours upon TNF-\( \alpha \) (Figure 14). Western blot analysis of TNF-\( \alpha \)-stimulated RASFs confirmed induction of SIRT1 protein expression after 48 hours (Figure 15B). Similarly to RASFs, activation of monocytes by TNF-\( \alpha \) resulted in a 4.8-fold increase of SIRT1 mRNA levels after 4 hours of stimulation (p<0.05), whereas at 24 hours SIRT1 mRNA levels were back to baseline (Figure 15C).

**Figure 14: Time course analysis of TNF-\( \alpha \)-induced SIRT1 expression.**
RASF (n=5-8) were treated with 10 ng/ml TNF-\( \alpha \) for 1, 4 and 8 hours. Levels of SIRT1 mRNA were measured by RT-PCR and are shown as fold induction relative to untreated cells [x-fold]. * = p<0.05, by ANOVA followed by Dunnett\( \alpha \) post-test.
Proinflammatory and antiapoptotic effects of SIRT1

Figure 15: SIRT1 is induced upon stimulation with TNF-α
(A) RASFs were treated with different TLR ligands (300 ng/ml Pam3CSK4, 10 μg/ml poly I:C, 10 ng/ml LPS) and two major RA cytokines (10 ng/ml TNF-α, 1 ng/ml IL-1β) for 24 hours (n=7-9). Changes in SIRT1 mRNA levels relative to untreated RASFs are shown [x-fold]. (B) Representative Western blots confirming induction of SIRT1 protein upon stimulation of RASFs with TNF-α for 48 hours (n=3). (C) Monocytes from healthy individuals (n=5-8) were treated with 10 ng/ml TNF-α for the indicated time points and SIRT1 mRNA levels relative to untreated cells are shown [x-fold]. * = p<0.05, by ANOVA followed by Dunnett’s post-test.

SIRT1 mediates apoptosis resistance in RASF.
As SIRT1 has been shown to prolong cellular life span and as spontaneous apoptosis is known to be reduced in activated RASFs, we assessed the effect of SIRT1 on apoptotic cell death. RASFs were transfected with siRNA and expression vectors as described above. SIRT1 silenced RASFs showed an increase of 48 and 41 % in the number of AnnexinV positive (p<0.02) and PI positive (p<0.03) cells, respectively. When RASFs were transfected to overexpress a vector encoding SIRT1, cells were less susceptible to apoptosis, displayed by a decrease in AnnexinV positive (49 %, p<0.05) and PI positive (46 %, p<0.05) RASFs (Figure 16). These results suggest that the constitutive overexpression of SIRT1 in RASFs may lead to a prolonged lifespan of these cells.

Figure 16: SIRT1 mediates apoptosis resistance in RASFs.
RASFs were transfected with SIRT1 specific siRNA (n=7) or with a vector encoding wild type SIRT1 (n=3) for 72 hours. Flow cytometry was used to measure levels of AnnexinV and PI positive cells relative to control siRNA (ctr) or mock (empty vector) transfected RASFs. Values are relative changes in the number of AnnexinV or PI positive cells compared to control transfected cells [x-fold], * = p<0.05, by paired t-test.
SIRT1 positively affects production of the proinflammatory mediators IL-6 and IL-8 in RASFs.

We next assessed whether SIRT1 also has a direct impact on the inflammatory phenotype of RASFs. Therefore, the production of the proinflammatory cytokine IL-6 was analysed in LPS stimulated RASFs after transfection with a SIRT1 expression vector. Interestingly, overexpression of SIRT1 increased the production of LPS induced IL-6 compared to mock transfected RASFs by 56 % (p<0.05). This enhancing effect on IL-6 production was not seen when cells were transfected with a mutant form of SIRT1 (H363Y, Figure 17A). Similar results were obtained by stimulation with TNF-α instead of LPS (Figure 18). Analysis of SIRT1 mRNA levels revealed successful overexpression in both wild type and mutant SIRT1 transfected RASFs (p<0.05, Figure 19).

![Graphs showing cytokine production](image_url)

**Figure 17:** Increased levels of proinflammatory cytokines in RASFs and monocytes overexpressing SIRT1. (A) RASFs were transfected with expression vectors encoding wild type (n=8) and mutant (n=8) forms of SIRT1. IL-6 protein concentrations were measured in culture supernatants at 40 hours post-transfection. (B) RASFs (n=5) were transfected with control siRNA (ctr) or SIRT1 specific siRNA (si1) for 48 hours and stimulated with 10 ng/ml LPS for another 24 hours. Basal and LPS induced production of IL-6 and IL-8 was measured in culture supernatants. (C) Primary peripheral monocytes from healthy individuals were transfected to overexpress wild type or mutant SIRT1 (n=8) and LPS induced TNF-α production in the supernatants was measured. (D) Monocytes (n=6) were transfected with control siRNA (ctr) or with SIRT1 siRNA (siSIRT1). LPS induced production of TNF-α in culture supernatants is shown. Values are means ± SEM. * = p<0.05, by paired t-test.
Accordingly, when RASFs were transfected with control or SIRT1 specific siRNA for 48 hours, there was a significant reduction of both basal and LPS stimulated expression of IL-6 and IL-8 after knockdown of SIRT1. Basal IL-6 and IL-8 production was diminished by 37 ± 11 % and 47 ± 21 % (p<0.05), respectively, whereas LPS induced IL-6 and IL-8 levels were reduced by 40 ± 15 % and 70 ± 14 % (p<0.05), respectively (Figure 17B). Also, IL-6 and IL-8 mRNA levels were reduced after SIRT1 knockdown, arguing against purely translational effects of SIRT1 (Figure 20). Successful knockdown of SIRT1 was confirmed by Real-time PCR (Figure 19B).

Figure 18: Increased levels of TNF-α induced IL-6 production in RASFs overexpressing SIRT1.
RASFs (n=9) were transfected with SIRT1 wild type expression vector or an empty vector control (mock) and stimulated with 10 ng/ml TNF-α IL-6 protein production was measured in culture supernatants at 40 hours post-transfection. Induction in IL-6 production is displayed relative to mock transfected cells. Values are means ± SEM. * = p<0.05, by paired t-test.

Figure 19: Confirmation of successful transfection with wild type or mutant SIRT1 and siRNA targeting SIRT1 in primary monocytes and RASFs.
RASFs were transfected with vectors encoding wild type and mutant SIRT1 (A) or SIRT1 specific siRNA (B). Monocytes from healthy individuals were transfected with wild type or mutant SIRT1 plasmids (C) or siRNA targeting SIRT1 (D).
SIRT1 mRNA relative expression units are presented as the difference in threshold cycle [delta Ct], relative to 18S rRNA. Values are means ± SEM. * = p<0.05, by paired t-test.
Results

**Figure 20: Knockdown of SIRT1 reduced mRNA levels of proinflammatory cytokines.**

RASFs (n=3) were transfected with control siRNA (ctr) or with SIRT1 siRNA (siSIRT1) for 48 hours and then stimulated with 10 ng/ml LPS for another 24 hours. Basal and LPS induced relative changes in mRNA levels for IL-6 (A) and IL-8 (B) are shown, compared to control transfected cells [x-fold].

Values are means ± SEM. * = p<0.05, by paired t-test.

**SIRT1 increases production of TNF-α in monocytes.**

As monocytes produce only low amounts of IL-6, we measured the production of TNF-α, a major cytokine involved in the pathogenesis of RA. Freshly isolated monocytes were subjected to transfection with SIRT1 expression vectors or siRNA as described above and subsequently stimulated with LPS. Consistent with the results obtained in RASFs, monocytes overexpressing wild type SIRT1 showed an increase of 36 % in TNF-α production compared to mock transfected cells (p<0.01). This induction in TNF-α levels was not seen when monocytes were transfected with the mutant form of SIRT1 (H363Y, Figure 17C). Non-stimulated cells did not produce detectable amounts of TNF-α. Further confirmation that SIRT1 signaling regulates the LPS induced production of TNF-α in monocytes was obtained by specific down-regulation of SIRT1 using siRNA. Measurement of LPS induced TNF-α in the cell culture supernatants indicated a significant decrease of 31 % (p<0.02, Figure 17D). Real-time PCR analysis verified successful transfections (p<0.02, Figures 19C and D).

**Enzymatic sirtuin inhibitors decrease TNF-α production in monocytes.**

As we found a prominent difference in the levels of TNF-α between wild type and mutant SIRT1 transfected monocytes, we tested the effects of commercially available SIRT inhibitors on TNF-α production. Monocytes were stimulated with LPS in presence of the SIRT1 specific enzymatic inhibitor EX-527 and the levels of TNF-α were measured. Consistent with our previous results, EX-527 reduced the expression of TNF-α by 37 ± 17 % (p<0.01, Figure 21A). Furthermore, we used the pan sirtuin inhibitor Sirtinol to block the activity of all the sirtuins. Interestingly, also Sirtinol reduced the LPS induced TNF-α production by 49 ± 9 % (p<0.01), suggesting that the sirtuin family overall has proinflammatory effects in monocytes (Figure 21B).
Figure 21: Inhibition of the activity of SIRT1 by enzymatic inhibitors results in reduced levels of TNF-\(\alpha\) (A) Monocytes from healthy individuals (n=9) were stimulated with 10 ng/ml LPS for 24 hours in presence or absence of the SIRT1 specific inhibitor EX-527 (9 \(\mu\)M). Concentrations of TNF-\(\alpha\) were measured in the culture supernatants (B) Monocytes (n=7) were stimulated with 10 ng/ml LPS in presence of the pan sirtuin inhibitor Sirtinol (30 \(\mu\)M) for 24 hours and the levels of TNF-\(\alpha\) were measured. Results are shown as means ± SEM. * = p<0.05, by paired t-test.

The proinflammatory activity of SIRT1 is mediated through NF-\(\kappa\)B dependent pathways.

NF-\(\kappa\)B is known to be essential for cytokine signaling in RA.[199] Additionally, SIRT1 was shown to affect expression of NF-\(\kappa\)B dependent genes, such as p53 and Bcl-2 [164, 200]. We assessed whether the effects of SIRT1 in monocytes are dependent on NF-\(\kappa\)B by using an inhibitor of IKK-2, sc-514. When SIRT1 was overexpressed in monocytes, LPS induced TNF-\(\alpha\) levels were increased as shown before. This SIRT1 dependent increase was completely blocked by treatment with the IKK-2 inhibitor sc-514, suggesting that SIRT1 acts via a NF-\(\kappa\)B dependent mechanism (Figure 22).

Figure 22: SIRT1 mediates its proinflammatory effects through NF-\(\kappa\)B. Primary human monocytes from healthy volunteers (n=4) were transfected to overexpress wild type SIRT1 or were mock transfected and stimulated with 10 ng/ml LPS in presence or absence of an inhibitor of IKK-2, sc-514 (50 \(\mu\)M, in DMSO). The production of TNF-\(\alpha\) was measured in the culture supernatants. Results are shown as means ± SEM. * = p<0.05, by paired t-test.
DISCUSSION

SIRT1, a member of the NAD+ dependent class III HDACs, deacetylates both histones and non-histone targets [193]. Thereby, SIRT1 controls a broad range of cellular processes, including cell survival and inflammation [173, 201]. Interestingly, SIRT1 was shown to be induced by calorie restriction (CR), thereby connecting SIRT1 with the beneficial effects of CR on longevity and age-related disorders such as diabetes [202]. In addition, increased levels of SIRT1 were found in diseases such as neurodegeneration, cancer and experimental autoimmune encephalomyelitis [203]. We have analysed SIRT1 expression and found SIRT1 to be overexpressed in RA synovial tissues as compared to OA. SIRT1 is expressed in synovial fibroblasts and monocytes/macrophages, suggesting that in joints SIRT1 is predominantly expressed in tissue resident cells.

The regulation of SIRT1 expression is incompletely understood. Different effects of cytokines on SIRT1 expression have been described. Both the cytokines IL-1β and interferon-γ have been shown to reduce the expression of SIRT1 in rat islets [204], whereas TNF-Î¼ induced SIRT1 expression in human vascular smooth muscle cells [205]. Here we show that the basal overexpression of SIRT1 in RA can be further induced upon stimulation with TNF-Î¼, a major cytokine found in joints of RA patients. However, SIRT1 expression was not influenced by stimulation with TLR ligands and IL-1β in vitro, suggesting that TLR pathways do not directly regulate SIRT1 expression.

SIRT1 has emerged as a key anti-aging protein in different experimental models, such as in *Sacharomyces cerevisiae*, in sirt1-null mice as well as in various in vitro cell cultures [164, 168, 172, 206-209]. RASFs characteristically show a resistance to apoptosis [54]. We have analyzed the effect of SIRT1 on apoptosis in RASFs. We found that overexpression of SIRT1 protected cells from apoptosis. The antiapoptotic effect of SIRT1 together with its constitutive high expression levels in RA suggest that SIRT1 may contribute to the apoptosis resistant phenotype seen in RASFs.

Up to now, published data on the effects of SIRT1 on inflammation is controversial. Macrophages from myeloid SIRT1 knock out mice displayed increased NF-Î³B mediated inflammation in response to environmental stress, indicating an anti-inflammatory activity of SIRT1 [165]. In agreement, a sirtuin activator had an inhibitory effect on LPS induced inflammation in intraperitoneal murine macrophages [167]. However, the biological effects mediated by SIRT1 seem to differ between cell types. It has been shown that the beneficial effects of calorie restriction induced SIRT1 in white adipose tissue or skeletal muscle cells were not seen in liver tissues [210]. A previous report using RA synovial biopsy explants...
showed that treatment with nicotinamide, a non-specific inhibitor of sirtuins, resulted in a reduction of IL-6 and TNF-α secretion, suggesting that the sirtuin family may have proinflammatory effects [144]. In line with this finding, it was recently reported that the proinflammatory cytokine HMGB1 stimulated production of TNF-α through activation of TLR4 in periodontal cells. This TLR4 dependent enhanced expression of TNF-α was blocked when SIRT1 or NF-κB were inhibited [211]. As we have previously shown that TLR activation results in upregulation of NAD+ producing PBEF/Visfatin and as SIRT1 activity is NAD+ dependent [153, 189, 212, 213], it is conceivable that TLR4 stimulation of synovial cells during chronic arthritis induces SIRT1 activity and that SIRT1 in a NF-κB dependent fashion prolongs the life-span of RASFs and enhances the proinflammatory cytokine production. Endogenous TLR4 ligands such as heat shock proteins, HMGB1 and others have been described, however, it is not known which of these are disease relevant in RA [214]. In our detailed analysis of the effects of SIRT1 on cytokine production in RASFs and monocytes, we found that overexpression of SIRT1 had a modulatory effect on IL-6 and TNF-α production in RASFs and monocytes, respectively, resulting in a significant induction of these cytokines in a NF-κB dependent fashion. These enhancing effects on cytokine expression were not seen when cells were transfected with an enzymatically inactive mutant form of SIRT1, which would argue that higher wild type SIRT1 expression levels may support the production of proinflammatory cytokines. Interestingly, the pan sirtuin inhibitor Sirtinol also reduced proinflammatory cytokines similar to the SIRT1 specific inhibitor EX-527, suggesting that the overall inhibition of sirtuins may have an anti-inflammatory effect.

In summary, we report the overexpression of SIRT1 in RA synovial tissue and despite evidence for an anti-inflammatory role of SIRT1 in animal models of inflammation we show that SIRT1 directly enhances proinflammatory cytokine production of synovial cells. Cytokine production is further potentiated by an anti-apoptotic effect prolonging the life span of RASFs. Our results suggest that careful investigation of the cell and disease specific effects of sirtuins is necessary to delineate possible therapeutic uses of agents targeting these molecules.
CHAPTER III  Downregulation of microRNA-34a* in rheumatoid arthritis synovial fibroblasts promotes apoptosis resistance

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miR-34a* mediates apoptosis resistance via XIAP

ABSTRACT

Objective: We investigated the expression and the impact of the microRNA-34 (miR-34) family on apoptosis in synovial fibroblasts (SF) of rheumatoid arthritis (RA) patients.

Methods: Expression of the miR-34 family was analyzed by real-time PCR in SF with or without stimulation with Toll-like receptor (TLR) ligands, TNF-Û, IL-1ß, hypoxia and 5-azacytidine. Promoter methylation was studied by combined bisulfite restriction analysis. Overexpression and silencing of miR-34a and miR-34a* was used to analyze their effect on apoptosis by annexin V/PI staining. Production of XIAP (X-linked inhibitor of apoptosis protein) was analyzed by real-time PCR and immunohistochemistry. Reporter gene assay was used to study the signaling pathways of miR-34a*.

Results: Basal expression levels of miR-34a*, but not of miR-34a, miR-34b/b* and miR-34c/c*, were found to be reduced in SF from RA compared to osteoarthritis. Neither TNF-Û, IL-1ß, TLR ligands nor hypoxia altered miR-34a* expression. However, we identified the promoter of miR-34a/34a* to be methylated and showed that transcription of the miR-34a duplex is induced upon treatment with demethylating agents. Enforced expression of miR-34a* led to an increased rate of FasL and TRAIL mediated apoptosis in RASF. Moreover, levels of miR-34a* highly correlated with the expression of XIAP, which was found to be upregulated in RA synovial cells. Finally, our study identified XIAP as a direct target of miR-34a*.

Conclusion: Our data provide evidence for a methylation specific downregulation of pro-apoptotic miR-34a* in RASF. Decreased expression of miR-34a* results in upregulation of its direct target XIAP, thereby contributing to apoptosis resistance of RASF.
INTRODUCTION

The pathogenesis of RA is characterized by a marked proliferation of the synovial tissue, invading and destroying periarticular bone and cartilage. The major effector cells of cartilage degradation are synovial fibroblasts (SF) [215]. RASF produce high levels of destructive enzymes and proinflammatory cytokines upon activation [44]. A characteristic feature of RASF is their decreased susceptibility to spontaneous as well as FasL and TRAIL mediated apoptosis [124, 125]. Whereas activation of the Fas pathway is associated with increased JNK signaling, TRAIL was reported to act via inhibition of the PI3K/Akt molecular pathway [42]. Several factors have been identified to inhibit receptor mediated apoptosis in RASF, such as SUMO-1 [216], FLIP [217] and Mcl-1 [218]. However, the mechanisms leading to the characteristic apoptosis resistance of RASF are still incompletely understood.

MicroRNAs (miRNA, miR) have emerged as fine tuning regulators for diverse biological processes [196, 219]. During their biogenesis, miR genes are transcribed into primary miRNA (pri-miR) that get processed by Drosha and Dicer to generate miRNA duplexes consisting of a mature and a passenger miR (also called miR star, miR*) strand [175]. Some miRs have been reported as important regulators of cellular lifespan. Of these, miR-34a was identified as a modulator of apoptosis in different reports. However, existing data are controversial. On the one hand, miR-34a was shown to be an inducer of programmed cell death in colon cancer cell lines and mouse embryonic fibroblasts [178-180]. On the other hand, miR-34a protected cells, such as human B lymphocytes and a mouse tubular cell line, from apoptosis [181, 182].

Since alterations of apoptosis are a characteristic finding in the RA synovium, we have analyzed the expression of the miR-34 family in RASF. MiR-34a, miR-34b/b* and miR-34c/c* were not found to be differentially expressed between RASF and SF from patients with non-inflammatory osteoarthritis (OA). However, the passenger strand of miR-34a, miR-34a*, was significantly downregulated in SF from patients with RA compared to OA. We analyzed the effects of miR-34a* on apoptotic cell death and identified XIAP (X-linked inhibitor of apoptosis protein) as a direct target of miR-34a*. Finally, we show that low levels of miR-34a* correlates with high XIAP expression in synovial fibroblasts.
RESULTS

MATERIAL & METHODS

Patients and tissue preparation. Synovial tissue specimens were obtained from patients with RA and OA, after informed consent had been obtained from all patients (Department of Orthopedic Surgery, Schulthess Clinic, Zurich, Switzerland). All RA patients fulfilled the American College of Rheumatology criteria for the classification of RA [10]. For cell culture, tissues were digested and synovial fibroblasts were grown as previously described [196]. Cultures of SFs were subjected to experimental procedures from passages 4 to 9.

Stimulation assays. SF were plated in 12-well plates (5 x 10^4 cells/well) in 1 ml supplemented DMEM and stimulated for 2, 8 or 24 hours with 10 ng/ml tumor-necrosis factor alpha (TNF-α), 1 ng/ml interleukin-1β (IL-1β) (both R&D Systems, Abingdon, UK), 300 ng/ml bacterial lipopeptide Pam3CSK4 (bLP), 10 μg/ml poly(I:C) (both InvivoGen, San Diego, CA) or 10 ng/ml lipopolysaccharide (LPS, from Escherichia coli J5; List Biologicals, Campbell, CA). For methylation studies, 5-azacytidine (Sigma-Aldrich, Buchs, Switzerland) was added every day to the cell culture medium at a concentration of 0.1 or 1 μM for 7 days. For induction of hypoxia, fibroblasts were exposed to a humidified atmosphere containing 1 % (hypoxia) or 20 % (normoxia) O₂ volume/volume for 2, 6 or 20 hours.

RNA isolation, reverse transcription, and real-time polymerase chain reaction (PCR). For miRNA analysis, RNA was isolated from fibroblasts with the mirVana Isolation Kit (Applied Biosystems, Rotkreuz, Switzerland). Total RNA was reverse transcribed and analyzed in a real-time PCR reaction using miR-specific TaqMan primers (Applied Biosystems). The expression of let-7a served as endogenous control for relative quantification. For the analysis of mRNA expression, isolated RNA was DNase digested and reverse transcribed. Single-reporter real-time PCR was performed using the ABI Prism 7700 Sequence Detection system. Quantification of XIAP mRNA was performed by SYBR green real-time PCR. The primer sequences used for XIAP were forward 5’-AAT TGG GAA CCT TGT GAT CG-3’ and reverse 5’-AGG AAA GTG TCG CCT GTG TT-3’ Eukaryotic 18S ribosomal RNA was measured using a predeveloped primer/probe system (Applied Biosystems) and served as endogenous control. Results were analyzed using the comparative threshold cycle (delta Ct) method for relative quantification as described previously [220].

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**Transfection experiments.** SF were transfected with 100 nM of synthetic precursor molecules (pre-miR) or inhibitors (anti-miR) of miR-34a and miR-34a*, respectively, or negative controls for pre-miR and anti-miR (all Applied Biosystems) using Lipofectamine 2000 reagent (Gibco-Invitrogen, Basel, Switzerland) according to the manufacturer's protocol. Transfected SF were incubated at 37 °C for 72 hours for basal expression analysis or for 48 hours when subjected to further experiments. Successful transfection was confirmed by real-time PCR using miR-specific TaqMan primers as described above.

**Fluorescence activated cell sorting (FACS).** SF were transfected with pre- and anti-miRs as described above. After 48 hours, medium was changed and cells were left untreated or stimulated for 24 hours with 200 ng/ml FasL (Alexis, San Diego, CA) and 20 ng/ml TRAIL (R&D Systems, Abingdon, United Kingdom), respectively. Cells were detached with accutase (PAA Laboratories, Pasching, Austria) and stained with annexin V-FITC and PI with the Annexin-V-FLUOS Staining Kit (Roche, Mannheim, Germany). Cells were subsequently analyzed on a FACSCalibur flow cytometer. Data were processed using CellQuest software (BD Biosciences, San Jose, CA).

**Immunohistochemistry (IHC).** Tissues were fixed in paraformaldehyde and embedded in paraffin. Synovial tissue sections were deparaffinized and pretreated with 10 mM citrate buffer. After blocking endogenous peroxidase and nonspecific binding, slides were incubated with mouse-anti-human XIAP antibodies (1:200, clone 117318, R&D Systems) or mouse IgG1 isotype control (Jackson, Suffolk, UK) over night at 4 °C. Sections were incubated with biotinylated goat-anti-mouse antibodies (Jackson) followed by incubation with HRP-conjugated streptavidin complex (ABC Kit, Vector laboratories, Peterborough, UK). XIAP positive cells were visualized using AEC. All sections were counterstained with hematoxylin.

**Combined bisulfite restriction analysis (COBRA) of the miR-34a/34a* promoter.** Genomic DNA was prepared from RASF and OASF using the QIAamp DNA blood Mini kit and bisulfite modified using the EpiTect bisulfite kit (both Qiagen, Hombrechtikon, Switzerland). PCR amplification was performed using Hot Start PCR and AmpliTaq Gold polymerase (Applied Biosystems). Primers were designed for the CpG area upstream of the miR-34a/34a* promoter as follows: hemi-forward: 5ʹGGG GAT TGT AGT GTT AGT TTT TTT-3ʹ forward: 5ʹTGT TGG TTT GGT TTT TGG ATT TTA-3ʹ reverse: 5ʹAAA AAA
TCA ACA CTT CCC TAA AAA AA-3' The PCR product was TaqI digested for 5 hours and analyzed on an agarose gel.

**Cloning of XIAP 3’UTR expression plasmids.** The 6800 bp three prime untranslated region (3’UTR) of XIAP was cloned into the pGL3control vector (Promega, WI, USA) in three overlapping parts. For PCR, human genomic DNA (Promega) and the following primers were used: XIAP 3’UTR part 1 (1-2299bp): forward: 5'GTA ATT CTA GAT CTA ACT CTA TAG TAG GCA TGT TA-3' reverse: 5'TAA ATT TAA AGT CTA GAC TAT ACA GAC CAA ATT C-3' XIAP 3’UTR part 2 (2249-4652bp): forward: 5'AGC TCG CTA GCC TGC CAC TTA GTT TGG TTA TAT AG-3' reverse: 5'CCG ACG CTA GCC ACA TTG TGT TAA CTG TAT GAG TC-3' XIAP 3’UTR part 3 (4579-6790bp): forward: 5'TCG AGT CTA GAG AGC TTT CTA AGA GAA GCA ATT GG-3' reverse: 5'CCG ACT CTA GAA ATT TTA AAG AAT AGT ATT TTA-3' The PCR products were gel-purified, digested with XbaI (part 1 and 3) or NheI (part 2) and inserted into the pGL3control plasmid via the XbaI restriction site downstream of the luciferase gene. The constructs were analyzed for correctness by sequencing.

**Reporter gene assay.** HEK293 cells were co-transfected with the 3’UTR constructs, the pRL_SV40 vector (Promega) as internal control, and the synthetic pre-miR-34a* or pre-control oligonucleotide using Lipofectamine 2000 reagent (Invitrogen). Firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay System and normalized to the activity of Renilla luciferase.

**Statistical analysis.** Unpaired and paired t-tests were used where appropriate for statistical evaluation of the data by GraphPad Prism 5.0 software. If not otherwise indicated, values are presented as mean ± SEM. p-values less than 0.05 were considered as significant.
RESULTS

Reduced constitutive expression levels of miR-34a* in RASF.

Since the miR-34 family was reported to influence apoptotic cell death in several studies, we performed expression analysis of miR-34a, miR-34b, miR-34c and of the corresponding passenger strands in synovial fibroblasts. Constitutive expression levels differed between individual miRs, which may be attributable to their varying chromosomal locations. MiR-34a has been shown to be present on chromosome 1, while miR-34b and c are located on chromosome 11. The expression of miR-34a as well as of miR-34b and c were not found to be significantly altered in RASF (n=7-20) compared to OASF (n=6-18). No differences in expression between SF from RA (n=5) and OA (n=5) patients were observed for the passenger strands of miR-34b and miR-34c. However, the passenger strand miR-34a* revealed a strong difference in constitutive expression levels between RASF (n=8) and OASF (n=10, Figure 23). Levels of miR-34a* were downregulated by 44 % in RASF (delta Ct: 5.96 ± 0.51) as compared to OASF (delta Ct: 5.13 ± 0.71, p<0.001).

![Figure 23: Decreased constitutive expression of miR-34a* in RASF](image)

Basal expression levels of miR-34a* (n=8-10), miR-34a (n=18-20), miR-34b* (n=5), miR-34b (n=6-7), miR-34c* (n=5) and miR-34c (n=8-10) in SF from patients with RA or OA are shown. Results are the differences in comparative threshold cycle (delta Ct) between the indicated miR and the internal control, let-7a. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the minimum and maximum values.

* = p<0.05, by unpaired t-test.
Methylation specific inactivation of miR-34a*.

To study the regulation of miR-34a* expression, we stimulated RASF with either TNF-\(\tilde{\text{U}}\) (n=7), IL-1\(\beta\) (n=4) or the TLR ligands bLP (TLR2, n=4), pIC (TLR3, n=5) and LPS (TLR4, n=4). Real-time PCR analysis showed no changes in miR-34a* expression upon cell activation at 24 hours in RASF (Figure 2A) as well as in OASF (data not shown). Time course analysis of TNF-\(\tilde{\text{U}}\) treated cells revealed no influence of this cytokine on miR-34a* expression also at 2 and 8 hours of stimulation (data not shown). During the establishment of RA, hypoxia occurs in the synovium [221]. To assess whether changes in the oxygen supply could alter the expression of miR-34a*, we incubated RASF (n=6) under hypoxic conditions for 2, 6 and 20 hours. Subsequent real-time PCR showed reduced miR-34a* levels after 20 hours of hypoxia, however, without reaching statistical significance (Figure 2B).

Since the mature miR-34a strand was previously shown to be regulated by DNA methylation [180, 222] and RASF display alterations in their DNA methylation pattern [136], we tested the influence of DNA demethylating agents on the expression of miR-34a and its passenger strand. RASF (n=6) were treated for one week with 0.1 or 1 uM 5-azacytidine (5-aza). MiR-34a and miR-34a* were both dose dependently induced (34 and 70 %, respectively) upon demethylation with 1 uM 5-aza, but statistical significance was only reached for miR-34a* (p<0.05, Figure 2C). In fact, combined bisulfite restriction analysis showed that the CpG island upstream of the promoter of miR-34a/34a* is methylated in RASF (Figure 2D), indicating that the expression of miR-34a and its passenger strand is under epigenetic regulation.

Pro-apoptotic effect of miR-34a* in RASF.

To investigate the role of miR-34a and miR-34a* in apoptosis of RASF, we overexpressed each miR using synthetic pre-miRs. Successful overexpression was confirmed by real-time PCR. Basal cell death was not significantly changed by transfection with pre-miRs (data not shown). Interestingly, we observed opposite effects of miR-34a and of its passenger strand on FasL mediated apoptosis (Figure 25A). Overexpression of miR-34a decreased the number of FasL stimulated, annexin V positive cells compared to control transfected RASF by 20 % (n=5, p<0.05), whereas cells transfected with pre-miR-34a* were more susceptible to FasL induced apoptosis, demonstrated by an increase from 25 % to 40 % in annexin V positive cells (n=6, p<0.05). In consistence, overexpression of miR-34a* further promoted TRAIL induced apoptosis from 22 % to 30 % (n=6, p<0.05, Figure 25B).
miR-34a* mediates apoptosis resistance via XIAP

Figure 24: Epigenetic inactivation of miR-34a and miR-34a* in RASF

(A) Expression of miR-34a* after stimulation for 24 hours with tumor necrosis factor (TNF-α, n=7), bacterial lipopeptide (bLP, n=4), poly(I:C) (pIC, n=5), lipopolysaccharide (LPS, n=4), and interleukin-1β (IL-1β, n=4). Real-time PCR was used to measure the expression. Results are normalized to let-7a and presented as x-fold changes relative to untreated RASF. (B) RASF (n=6) were cultured for 20 hours under normoxia (20% O2) or hypoxia (1% O2). Levels of miR-34a* expression were measured by real-time PCR and normalized to let-7a. Results are presented as differences in comparative threshold cycle (delta Ct). (C) Detection of expression levels of miR-34a* and miR-34a in untreated, 0.1 or 1 μM 5-azacytidine (5-aza) stimulated RASF (n=6). Expression was measured by real-time PCR after 1 week of treatment and normalized to let-7a. Results are presented as differences in comparative threshold cycle (delta Ct). (D) Bisulfite modified DNA isolated from SF from patients with RA (n=3) was PCR amplified using primers recognizing the CpG island upstream of the miR- 34a/34a* promoter. The PCR products were left untreated (U) or were digested (D) using TaqI. *TaqI recognizes the sequence TCGA and therefore only cleaves when the CpG sequence has been preserved by a methylated cytosine during bisulfite conversion. DNA was visualized using agarose gel electrophoresis. The restricted bands demonstrate that the promoter of miR-34a/34a* is methylated in these cells. * = p<0.05, by paired t-test.
Transfection of miR-34a did not change levels of TRAIL mediated apoptosis. Thus, the results suggest that the constitutively low expression levels of miR-34a* in RASF result in decreased apoptosis. In accordance with the data obtained with pre-miR-34a*, silencing of miR-34a* in RASF resulted in reduced level of apoptosis, verified by a decrease from 19% to 17% in annexin V positive cells (n=4, p<0.05, Figure 25C). When OASF overexpressed miR-34a*, apoptosis was further increased from 13% to 20%, similar to the results obtained with RASF (n=8, p<0.05, Figure 25D).

Figure 25: miR-34a* induces apoptotic cell death in RASF
RASF (n=5-6) were transfected with synthetic pre-miRs to overexpress miR-34a (pre-34a), miR-34a* (pre-34a*) or non-specific control miR (pre-ctr). 48 hours post transfection, cells were stimulated with 200 ng/ml FasL (A) and 20 ng/ml TRAIL (B) for 24 hours and analyzed for annexin V binding by flow cytometry. (C) RASF (n=4) were treated with anti-miR-34a* (anti-34a*) or non-specific control miR (anti-ctr) for 48 hours. Cells were stimulated with 20 ng/ml TRAIL for 24 hours and subsequently analyzed for annexin V binding. (D) OASF (n=8) were transfected with pre-34a* to overexpress miR-34a* or with pre-ctr as transfection control for 48 hours. Cells were stimulated with 20 ng/ml TRAIL for 24 hours and subsequently analyzed for annexin V binding.

* = p<0.05, by paired t-test.
XIAP mediates the pro-apoptotic effects of miR-34a*.

Based on target prediction algorithms (http://diana.cslab.ece.ntua.gr/), we identified XIAP as a potential target gene of miR-34a*. To assess a regulatory role of miR-34a* on the expression of XIAP, we transfected RASF with the precursor for miR-34a* (pre-34a*) or a non-specific pre-miR control and performed silencing experiments using anti-miRs targeting miR-34a* (anti-34a*) or non-specific control anti-miRs. When RASF were transfected with pre-miR-34a*, levels of XIAP were significantly reduced by 36% in comparison to control pre-miR transfected cells (n=13, p<0.05), whereas silencing of miR-34a* (anti-34a*) increased expression of XIAP in comparison to control anti-miR transfected RASF by 90% (n=5, p<0.05, Figure 26A). In addition, we studied the effect of the mature miR-34a strand on XIAP. Neither overexpression nor silencing of miR-34a altered production of XIAP (Figure 26A). Our findings suggest that miR-34a* exerts its pro-apoptotic effect via XIAP. To analyze the expression of XIAP in the synovium, we performed immunohistochemistry on RA synovial tissue sections. In accordance to what has been shown previously [223], we found XIAP to be abundantly expressed throughout the entire RA synovial tissue (n=4), whereas in synovial tissue specimens from patients with OA (n=4) XIAP was mainly found at perivascular areas (Figure 27).

![Image of Figure 26](image_url)

**Figure 26: Modulation of XIAP expression by miR-34a***

(A) RASF were transfected with pre-miRs (n=9-13) or anti-miRs (n=5) specific for miR-34a and miR-34a*, respectively, or negative pre-miR and anti-miR controls. Expression of XIAP mRNA was analyzed by real-time PCR using XIAP specific primers. Results are normalized to 18S rRNA and expressed as x-fold changes relative to the control transfected RASF. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the minimum and maximum values. (B) Basal expression of XIAP in SF from patients with RA and OA (n=4). Results are presented as the difference in threshold cycle (delta Ct), relative to 18S rRNA. (C) The correlation between basal levels of XIAP and constitutive expression of miR-34a* in synovial fibroblasts (n=11) was determined by nonparametric Spearman correlation.

* = p<0.05, by paired t-test (A), unpaired t-test (B) or Spearman correlation (C).
The results of the IHC were confirmed by quantitative real-time PCR on synovial fibroblasts. Constitutive levels of XIAP were 2.1 fold higher in RASF (delta Ct: 11.45 ± 0.57) compared to OASF (delta Ct: 12.54 ± 1.67, n=5-6, p<0.05, Figure 26B). Furthermore, basal expression of XIAP mRNA was highly correlated with constitutive miR-34a* production (n=11, Spearman r=0.8, p<0.005, Figure 26C). These data suggest that the constitutive downregulation of miR-34a* in RASF is responsible for the upregulation of XIAP.

**Figure 27: XIAP expression in synovial tissues from patients with RA and OA**
Representative sections of synovial tissue specimens from patients with RA and OA stained for XIAP (red). Sections were counterstained with hematoxylin (blue). One representative section for positive XIAP staining in both RA and OA tissues, and one representative section of mouse IgG1 negative control staining of RA are shown (n=4). Insets designate areas further magnified below. Original magnification x100 or x400, as indicated.

**XIAP is a direct target of miR-34a*.**
To test whether XIAP is a direct target of miR-34a*, we performed a reporter gene assay. Due to its long sequence, the 3'UTR of XIAP was divided into three overlapping parts and each fragment was cloned into the pGL3control vector (Figures 28A and B). Co-transfection of HEK293 cells with the XIAP 3'UTR construct 1 and pre-miR-34a* yielded a 45 ± 8 % lower relative luciferase activity as compared to control transfected cells (p<0.002, n=4, Figure 28C). When HEK293 cells were transfected with the XIAP 3'UTR constructs 2 or 3, no reduction in the relative luciferase activity was observed after transfection with pre-miR-34a*. These findings indicate a direct regulatory interaction of miR-34a* with the apoptosis inhibitor XIAP via binding to the first part of the XIAP 3'UTR.
miR-34a* mediates apoptosis resistance via XIAP

Figure 28: XIAP is a direct target of miR-34a*

(A) Due to its size, the 3’ untranslated region (3’UTR) of XIAP was divided into three overlapping parts XIAP 1, XIAP 2 and XIAP 3. Putative miR-34a* seed matches were identified by Diana Lab algorithms (http://diana.cslab.ece.ntua.gr/) and their position inside the XIAP 3’UTR are indicated.

(B) Each part of the 3’UTR of XIAP was cloned downstream of the luciferase gene which is under the control of the constitutively active SV40 promoter.

(C) Reporter gene assay of HEK293 cells (n=4) co-transfected with luciferase constructs containing the XIAP 3’UTR (XIAP 1, 2 or 3) downstream of the luciferase gene and pre-miR-34a* or negative pre-control. Data are represented as relative luciferase activity. * = p<0.002, by paired t-test.
DISCUSSION

The prolonged cellular life span is a fundamental characteristic of activated synovial fibroblasts contributing to chronic inflammation and joint destruction in RA. In the current study we focused on the expression and function of miR-34, a family of microRNAs that is known to influence apoptotic cell death [178, 182, 224-226]. Whereas miR-34a, miR-34b/b* and miR-34c/c* did not show any significant differences in their expression levels, we found a significant downregulation of the passenger strand miR-34a* in RASF compared to OASF. Although microRNAs have highly attracted researchers’ attention in the last years, still little is known about the relevance of miR passenger strands. Some years ago, it was proposed that the regulation of the expression and accumulation of miR/miR* duplexes represents a tissue dependent mechanism [176]. There is evidence for an active sorting of both miR and miR* species into regulatory complexes, arguing for a biological role of miR* strands [227]. For instance, the passenger strand of miR-17 was shown to directly target the p50 subunit of NF-κB in a human cell line for malignant pleural mesothelioma [228]. In addition, miR-30* was able to bind and suppress the translation of target genes, suggesting that both the mature and the passenger strands are functional [176]. With respect to our studies, the passenger strand miR-34* was found to be localized to AGO1, a protein crucial for miRs to exert their biological functions [227, 229].

Recently, our group reported alterations in the DNA methylation status between synovial cells from patients with RA and OA [136]. In the present study, we demonstrated that the expression of miR-34a and its passenger strand was increased upon demethylating treatment, although statistical significance was only reached for miR-34a*. In accordance with our data, miR-34a expression was found to be silenced in various cancers due to aberrant CpG methylation [180, 224]. In contrast, cytokines such as TNF-Î” and IL-1Î² as well as TLR ligands did not influence the expression of miR-34a*.

Whereas impaired apoptosis of synovial fibroblasts is important for the pathogenesis of RA, little is known about the miR-dependent regulation of apoptosis. In agreement with previous data, our present findings suggest that both miR-34a and miR-34a* are involved in the regulation of apoptotic pathways. We present here evidence that miR-34a* promotes apoptosis in both FasL and TRAIL stimulated RASF, whereas overexpression of the mature strand miR-34a protected cells from FasL mediated apoptosis but had no effect on TRAIL induced cell death. Controversial data are published on the effect of miR-34a on apoptosis. In the colon cancer cell line HCT116, miR-34a was found to promote apoptosis [178]. Similarly, miR-34a was found to suppress SIRT1, ultimately leading to p53-dependent apoptosis in
cancer cells [225]. However, a recent study reported anti-apoptotic effects of miR-34a in B lymphoid cells [182]. In agreement with this finding, upregulation of miR-34a showed a positive association towards a longer survival of lung cancer cells [224]. Concerning the passenger strand miR-34a*, no data on its effect on apoptosis have been published before. In our study, we demonstrate opposite effects on apoptotic cell death of miR-34a and miR-34a* with the passenger strand increasing FasL and TRAIL induced apoptosis of RASF. Similarly, such a controversial outcome was observed for miR-155 and its corresponding passenger strand in terms of modulation of the expression of interferon-Ó [177].

Furthermore, we found miR-34a* to mediate its pro-apoptotic effects upon targeting X-linked inhibitor of apoptosis (XIAP). XIAP is a member of the IAP family of apoptosis inhibitors that block apoptosis by direct binding to caspases, leading to the inhibition of the execution phase of apoptosis [230]. It was recently shown that XIAP is implicated in innate immunity by mediating NOD1/2 signaling [231, 232]. In agreement with our findings, higher expression levels of XIAP were found in synovial tissues from active RA compared with normal individuals [223] and, in addition, XIAP was reported to be overexpressed in synovial fluid from juvenile idiopathic arthritis patients [233]. Induction of XIAP was observed upon stimulation with hematopoietic cytokines like granulocyte macrophage colony-stimulating factor, granulocyte colony-stimulating factor and stem cell factor in a human leukemia cell line [234]. Here, we present evidence for a new regulatory role of miR-34a* in the expression of XIAP. Basal expression levels of XIAP correlated with the constitutive miR-34a* production and overexpression/inhibition of miR-34a* modulated XIAP production in RASF. Using reporter gene assays, we showed that miR-34a* directly targets XIAP via binding to its 3Ã calendar UTR region. To our knowledge, this is the first report of an effect of the passenger strand miR-34a* on apoptosis. Our data indicate a functional impact of miR* species which should be taken into account when analyzing the role of miRs in the pathogenesis of RA.

In conclusion, we provide evidence for a pro-apoptotic role of miR-34a* that is mediated by the modulation of the expression of XIAP. As miR-34a* is downregulated in RASF, unopposed expression of XIAP may importantly contribute to the apoptosis resistance of these cells.
CHAPTER IV  CONCLUSION AND OUTLOOK
CONCLUSION AND OUTLOOK

In this work, we show that the high levels of SIRT1 found in RA synovial cells and tissues protect cells from apoptosis. Thus, SIRT1 contributes to the crucial phenotype of apoptosis resistance seen in RASF. Furthermore, we present a modulatory effect of SIRT1 on proinflammatory cytokine production in RASF and monocytes. Upon overexpression of SIRT1, production of IL-6 and TNF-α were increased in a NF-κB dependent fashion (Figure 29), whereas silencing of SIRT1 using different approaches reduced levels of these cytokines.

Figure 29: SIRT1 contributes to joint inflammation in RA.
Overexpression of SIRT1 in synovial cells from patients with RA contributes to the cellular longevity and to the NF-κB dependent production of pro-inflammatory cytokines such as TNF-α IL-6 and IL-8. NF-κB resides in the cytoplasm in an inactive form associated with IκB. Upon phosphorylation of IκB, NF-κB is released for nuclear translocation to alter gene expression.

In Chapter III of this thesis, we demonstrate down regulation of the passenger strand of miR-34a in synovial fibroblasts from patients with RA. In line with previous reports, we confirm methylation dependent regulation of the expression levels of miR-34a* [180]. Since RASF show an aberrant DNA methylation pattern, it might be speculated that epigenetic alterations are responsible for the low levels of miR-34a* found in the RA synovium.
Reduced amounts of miR-34a* were observed to protect cells from TRAIL and FasL induced apoptosis. We identified the apoptosis inhibitor XIAP as a direct target of miR-34a* and in line with previous reports, we confirmed upregulation of XIAP in the RA synovium [223]. Via an increase in XIAP expression, down regulation of miR-34a* may ultimately result in diminished activation of caspases and thus reduction of apoptosis (Figure 30).

Rheumatoid arthritis is characterized by persistent synovial inflammation with infiltration of inflammatory cells into the synovial lining. Activated immune cells and resident synovial cells propagate inflammation via cellular crosstalk and the secretion of high amounts of cytokines and chemokines, recruiting more inflammatory cells into the joints. Synovial macrophages and fibroblasts secrete high amounts of IL-1, IL-6 and TNF-α[24]. Therapeutic benefits of modulation of TNF-α and IL-6 and to a lesser degree IL-1β further confirm their importance in the pathogenesis of RA. However, not all patients respond well to these treatments, indicating a genetic heterogeneity of the disease. Indeed, numerous studies identified several immune associated genetic risk factors for developing RA [25]. Intriguingly, the HLA locus, which contains a large number of genes related to adaptive
immunity, accounts for 30 to 50% of overall genetic risk for RA [26]. Gene polymorphisms in \textit{PTPN22}, which is involved in T cell activation, have been implicated in RA pathogenesis [235], suggesting that adaptive immunity plays an important role in the pathogenesis of RA. Beyond genetics, epigenetic alterations have emerged in recent years as an additional mechanism of control of gene regulation. Epigenetic processes induce stable modifications in gene expression that do not involve a change in the nucleotide sequence [5]. DNA methylation by DNMTs and histone deacetylation by HDACs account for the two major epigenetic mechanisms. It has recently been hypothesized that the aggressive phenotype of synovial fibroblasts from patients with RA may at least partly result from changes in the epigenome, since loss of promoter specific methylation marks increased the production of CXCL12 in RASF, ultimately resulting in enhanced expression of destructive MMPs [57, 136]. Changes in histone acetylation at promoters of specific genes, including apoptosis-repressing SUMO-1 and destructive MMP-1, have been observed to contribute to the chronicity of RA [236]. Published data on the global histone acetylation pattern in RA are controversial. On the one hand, HDAC activity was reported to be decreased in RA synovial tissues compared to OA (142). On the other hand, others found higher levels of HDAC activity in synovial tissues of RA patients than in OA patients, suggesting a global decrease in histone and non-histone acetylation [148, 237]. Up to now it is not clear whether and in what direction the acetylation pattern is changed in RA. But interestingly, inhibition of HDAC activity has proved to be effective in several arthritic animal models. Both the HDAC inhibitors FK-288 and TSA were described to inhibit joint swelling, synovial inflammation and articular destruction [146, 147]. Similarly, \textit{in vitro} treatment of RA macrophages and synovial explants showed potent anti-inflammatory action of HDAC inhibitors [144]. Specifically, nicotinamide reduced the production of proinflammatory cytokines as well as IL-10 in primary human macrophages, possibly via inhibiting sirtuins [144], suggesting that modulation of epigenetic regulation might have therapeutic relevance [238].

Another mechanism of posttranscriptional regulation of gene expression is represented by microRNAs. The small non-coding miRs emerged as crucial modulators of numerous pathways and were found to be dysregulated in cancer, autoimmune diseases and chronic inflammation [134]. In RA synovial cells, alteration in the miR expression pattern has been observed to promote inflammation [196, 239]. Numerous reports have documented on promising approaches to successfully modulate miR expression \textit{in vivo}. A single subcutaneous administration of miR-34a was observed to efficiently suppress tumour development in mice [240]. Adenoviral silencing of miR-103 and miR-107 in obese animals
enhanced insulin stimulated glucose uptake, thus implicating these miRs as therapeutic targets in the treatment of diabetes [241]. Injection of miR-15a directly into murine knee joints was reported to induce synovial cell apoptosis in an arthritic mouse model [242], and repeated intravenous administrations of miR-146a could prevent joint destruction in arthritic mice [243].

In summary, our data highlight an important role for epigenetic mechanisms in the regulation of inflammation and apoptosis. Overexpression of the histone deacetylase SIRT1 not only enhances levels of inflammation, but also confers resistance to apoptotic cell death. Similarly, the small non-coding microRNA-34a* has a promoting effect on the cellular lifespan of RA synovial cells and thus, contributes to the sustained production of inflammatory mediators.

Further studies on epigenetic mechanisms of gene expression control in inflammation will increase our understanding of the disease pathogenesis and hopefully lead to the identification of new therapeutic targets for the treatment of RA.
CHAPTER V

APPENDIX
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
<th>Description</th>
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<tbody>
<tr>
<td>3'UTR</td>
<td>three prime untranslated region</td>
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<tr>
<td>ACPA</td>
<td>anti-citrullinated protein antibody</td>
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<tr>
<td>ACR</td>
<td>american college of rheumatology</td>
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<tr>
<td>BCL-2</td>
<td>B cell lymphoma 2</td>
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<td>BH3</td>
<td>Bcl-2 homology domain 3</td>
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<td>CIA</td>
<td>collagen induced arthritis</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CCL</td>
<td>chemokine ligand</td>
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<tr>
<td>CTLA4</td>
<td>cytotoxic T lymphocyte antigen 4</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DAS</td>
<td>disease activity score</td>
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<tr>
<td>DISC</td>
<td>death-inducing signalling complex</td>
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<td>DMARD</td>
<td>disease-modifying antirheumatic drugs</td>
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<td>DNMT</td>
<td>DNA methyltransferases</td>
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<tr>
<td>DR</td>
<td>death receptor</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>ESR</td>
<td>erythrocyte sedimentation rate</td>
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<tr>
<td>EULAR</td>
<td>european league against rheumatism</td>
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<tr>
<td>FADD</td>
<td>Fas-associated protein with death domain</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<tr>
<td>GCP</td>
<td>granulocyte chemotactic protein</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony-stimulating factor</td>
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<td>gp130</td>
<td>glycoprotein 130</td>
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<tr>
<td>HAT</td>
<td>histone acetylase</td>
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<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<tr>
<td>HDACi</td>
<td>histone deacetylase inhibitor</td>
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</tr>
<tr>
<td>HIF-1Îº</td>
<td>hypoxia inducible factor 1 alpha</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>HSF-1</td>
<td>heat shock factor protein</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IL-1Ra</td>
<td>interleukin-1 receptor antagonist</td>
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<tr>
<td>IL-6RÎº</td>
<td>interleukin-6 receptor alpha</td>
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<tr>
<td>IRAK</td>
<td>interleukin-1 receptor-associated kinase</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>KDAC</td>
<td>lysine deacetylase</td>
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<tr>
<td>KDAC</td>
<td>lysine deacetylase</td>
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<tr>
<td>MCP</td>
<td>monocyte chemoattractant protein</td>
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<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
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<tr>
<td>miR</td>
<td>microRNA</td>
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<tr>
<td>miR*</td>
<td>microRNA passenger strand</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinases</td>
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<tr>
<td>MTX</td>
<td>methotrexate</td>
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<tr>
<td>NAD+</td>
<td>nicotinamide adenine dinucleotide</td>
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<tr>
<td>Nampt</td>
<td>nicotinamide phosphoribosyltransferase</td>
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<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drugs</td>
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<tr>
<td>OA</td>
<td>osteoarthritis</td>
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<tr>
<td>PADI4</td>
<td>peptidylarginine deiminase type 4</td>
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<tr>
<td>PARP</td>
<td>poly (ADP-ribose) polymerase</td>
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<tr>
<td>PBEF</td>
<td>pre-B cell colony-enhancing factor</td>
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<tr>
<td>PGE2</td>
<td>prostaglandin E2</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
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<tr>
<td>pre-miR</td>
<td>precursor microRNA</td>
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<tr>
<td>pri-miR</td>
<td>primary microRNA</td>
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<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
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<tr>
<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor γ</td>
<td></td>
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<tr>
<td>PTPN22</td>
<td>Protein tyrosine phosphatase, non-receptor type 22</td>
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<tr>
<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
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<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
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<tr>
<td>SCID</td>
<td>severe combined immunodeficient</td>
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<tr>
<td>SDF</td>
<td>stromal cell derived growth factor</td>
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<tr>
<td>SIRT</td>
<td>sirtuin</td>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitors of metalloproteinases</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
<td></td>
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<tr>
<td>TNF-Îº</td>
<td>tumour necrosis factor alpha</td>
<td></td>
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<tr>
<td>TNFAIP</td>
<td>TNF alpha induced protein</td>
<td></td>
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<tr>
<td>TRAIL</td>
<td>TNF related apoptosis inducing ligand</td>
<td></td>
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<tr>
<td>TSA</td>
<td>trichostatin A</td>
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</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
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CURRICULUM VITAE

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American College of Rheumatology (ACR), Chicago

August 2011  The passenger strand of microRNA-34a mediates apoptosis resistance via failure of XIAP (X-linked inhibitor of apoptosis protein) inhibition in RA synovial fibroblasts
Zurich Center for Integrative Human Physiology (ZIHP), Zurich
June 2011  Overexpression of SIRT1 inhibits apoptosis and promotes production of inflammatory mediators in synovial fibroblasts of patients with rheumatoid arthritis

*Day of Clinical Research, Zurich*

May 2011  Overexpression of SIRT1 inhibits apoptosis and promotes production of inflammatory mediators in synovial fibroblasts of patients with rheumatoid arthritis

*Oral presentation, European League Against Rheumatism (EULAR), London*

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*American College of Rheumatology (ACR), Atlanta*

August 2010  Inhibition of sirtuin activity reduces inflammatory and destructive mediators in rheumatoid arthritis synovial fibroblasts

*Zurich Center for Integrative Human Physiology (ZIHP), Zurich*

October 2009  Expression of sirtuins in rheumatoid arthritis synovial fibroblasts

*American College of Rheumatology (ACR), Philadelphia*

**Publications:**

Downregulation of microRNA-34a* in rheumatoid arthritis synovial fibroblasts promotes apoptosis resistance


*Arthritis & Rheumatism, 2011; DOI:10.1002/art.34334*

SIRT1 overexpression in the Rheumatoid Arthritis synovium contributes to proinflammatory cytokine production and apoptosis resistance


*Annals of the Rheumatic Diseases, 2011;70:1866-1873.*
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