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Somatostatin Regulates the Extracellular Regulated Kinase Cascade of Human Rheumatoid Arthritis Synoviocytes

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SOMATOSTATIN REGULATES THE EXTRACELLULAR REGULATED KINASE CASCADE OF HUMAN RHEUMATOID ARTHRITIS SYNOVIOCYTES

By

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ABSTRACT

Somatostatin (SRIF, somatotropin release inhibitory factor) is a ubiquitously expressed neuropeptide, interacting with cells via five SRIF receptor subtypes (sst_1-sst_5), belonging to the guanine nucleotide binding protein-coupled receptor (GPCR) superfamily. SRIF receptors have been well documented for their ability to inhibit cell proliferation and secretion in a variety of animal tissues. Over the past 15 years, pre-clinical and clinical studies implicate that SRIF and SRIF analog therapy improve symptoms in patients suffering from rheumatoid arthritis (RA). In this study, we investigate SRIF's effects on intracellular signaling in the synovium, the cellular layer that lines synovial joints, using *in vitro* cultures of human synovial fibroblasts. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of synovial isolated mRNA established the presence of sst_2 in these cells. The sst_2 receptor links somatostatin action to control of synovial intracellular signaling. We have examined SRIF effects on the synoviocyte extracellular regulated kinase pathway (ERK). Using phospho-specific antisera, we demonstrate that SRIF and SRIF analogs decrease phospho-ERK1/2 levels, suggesting basal ERK1/2 is upregulated in RA synoviocytes. SRIF also suppressed basal levels of activated Raf and MEK1/2, two upstream regulatory kinases of ERK1/2. Furthermore, SRIF suppressed TNFα-stimulated activation of ERK1/2 in RA synoviocytes. We also observed that SRIF increases a sodium vanadate-sensitive intracellular protein phosphatase activity in RA synoviocytes, indicating a possible intracellular mechanism by which SRIF controls the phosphorylation status of the ERK1/2 kinases. Fluorescent confocal scanning microscopy reveals that TNF-α stimulated the localization of phospho-ERK1/2 in the nuclei of RA synoviocytes, which was inhibited upon co-treatment with SRIF. Taken together our results demonstrate that SRIF regulates intracellular signaling in rheumatoid synoviocytes.

INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory, autoimmune disease that affects multiple organ systems and is of unknown etiology. In the affected joint, the synovicoyte is a major cellular target of pro-inflammatory mediators. Synoviocyte dysfunction is characteristic of RA and is defined by unregulated cellular hypertrophy and hyperplasia. In addition to synovial activation, angiogenesis and leukocyte infiltration ultimately lead to joint deterioration and reduced life expectancy (O'Dell, 2004; Choy and Panayi, 2001). Current RA therapies are not curative, however the disease modifying anti-rheumatic drugs (DMARDS) alleviate many RA symptoms (O'Dell, 2004). Clearly, a better understanding of RA synovial signal transduction would prove useful in developing long-term therapies that preserve synovial integrity while suppressing the chronic inflammation.

Although the initial immune trigger is unknown, CD4+ T-lymphocyte activation is an early event in RA pathology. Consequently, activated immune cells interact with the synovium and secrete pro-inflammatory cytokines. The synovium is comprised of both type A macrophage-like synoviocytes and type B fibroblast-like synoviocytes. Together with infiltrating T-lymphocytes, these three cell types dominate the RA synovial joint and are believed to mediate RA pathophysiology via intercellular signaling (Tran, *et al.*, 2005). Immune cytokine release includes interleukins-1 and -6 (IL-1, IL-6)

and tumor necrosis factor alpha (TNF-α), all of which initiate and promote synovial cytokine production and release (Choy and Panayi, 2001). TNF-α and IL-1β are the dominant pro-inflammatory mediators of this cytokine feedback loop which promotes the formation of an inflamed cellular pannus that induces chronic and aggressive joint destruction (Paran and Paran, 2003).

The body's endogenous hormones and peptides regulate a spectrum of immune events (Krantic, 2001). For example, vasoactive intestinal peptide (VIP), originally identified as a vasodilator, has documented anti-inflammatory effects in several inflammatory diseases including septic shock syndrome, Crohn's disease, and arthritis, mimicking a Th2 cytokine (Pozo and Delgado, 2004). Substance P, a peptide neurotransmitter involved in pain transmission and contraction of intestinal smooth muscle, plays a role in neuroinflammation and has been indicated as a mediator of multiple sclerosis pathophysiology (Reinke and Fabry, 2006). Somatostatin (SRIF) modulates endothelial inflammation and immune cell recruitment, hallmark events in coronary artery disease (Badway and Blake, 2005). Somatostatin controls a host of systemic activities related to cell proliferation and secretion. SRIF was originally identified as a hypothalamic neurohormone that inhibits the release of growth hormone (GH, somatotropin) from the adenohypophysis (Brazeau, *et al.*, 1973). Since then, SRIF has been found to control a spectrum of physiological functions, including the secretion of glucagon, insulin, thyroid stimulating hormone, and gastric acid, as well as the proliferation of normal and tumor cells (Dasgupta, 2004; Guillermet-Guibert, *et al.*, 2005). Indeed, SRIF has shown clinical utility in controlling the inappropriate

hypersecretion of growth hormone, hallmark of acromegaly (Melmed *et al.*, 2005). Due to its extensive involvement in regulating cellular secretion, a role for SRIF in chronic inflammatory conditions has been proposed.

SRIF peptides SRIF-28 and SRIF-14 are the predominant isoforms found in mammals (Reichlin, 1983), and are produced in the gut, CNS, and by tumor cells in a cell-specific manner (Patel, 1999). Cortistatin, a recently discovered member of the SRIF peptide family, appears to exhibit a discrete localization, while exhibiting a receptor binding pattern similar to the SRIF peptides (Fukusumi, *et al.*, 1997; Dalm, *et al.*, 2003; de Lecea, 2005). The regional distribution of mature SRIF peptides differ: SRIF-14 is widespread throughout the CNS and peripheral tissues while SRIF-28 is localized to the gut and cortistatin to the immune system (Reisine and Bell, 1995a; de Lecea, 2005). Both SRIF-14 and -28 originate from a single-gene product, a 116 amino acid prepro-somatostatin that is cleaved into its aforementioned active counterparts, SRIF-28 containing 14 additional residues at the amino terminus. Cortistatin is derived from a separate gene product, with a tissue-specific expression pattern (Dalm, *et al.*, 2003). The SRIF family of peptides exhibits a unique FWKT binding domain that is preserved in a β-turn configuration by an internal disulfide linkage (Patel and Reichlin, 1979). This binding domain is critical for receptor recognition (reviewed in ten Bokum, *et al.*, 2000), and is exploited in the design and use of biologically active peptidomimetics (Blake, *et al.*, 2004; Vaysse, *et al.*, 2005).

SRIF initiates its physiologic effects by ligating a family of highly homologous, heterotrimeric guanine nucleotide binding protein (G protein) coupled receptors (GPCRs)

designated sst_1-sst_5, including the splice variants sst_{2A} and sst_{2B}. The SRIF receptor subtypes exhibit a distinct yet overlapping expression in tissues (Reisine and Bell, 1995b; Krantic, 2001). SRIF receptors primarily couple to the inhibitory G_i and G_o proteins, which when activated, reduce cellular responsiveness (Hoyer, *et al.*, 1995), however the downstream signaling mechanisms that predicate the physiologic response are both tissue and receptor subtype specific. (Lahlou, *et al.*, 2004).

SRIF receptor subtype sst_2 is expressed in the synovium of patients with RA, identifying the synoviocytes as a cellular target for SRIF control (ten Bokum, *et al.*, 1999; Lu, *et al.*, 2001). SRIF actions *in vitro* on RA synoviocytes have been observed; SRIF inhibits cytokine release and proliferation in RA synoviocytes (Takeba, *et al.*, 1997). Clinically, SRIF has proven useful in treating RA. For instance, intra-articular administration of SRIF-14 proves analgesic in RA patients and reduces the synovial pannus thickness, as assessed by ultrasound (Fioravanti, *et al.*, 1995; Coari, *et al.*, 1995; reviewed in Paran and Paran, 2003). Synthetic, receptor subtype-selective SRIF analogs have proven especially effective (Matucci-Cerinic, *et al.*, 1992; reviewed in Paran and Paran, 2003), and recently intra-muscular injections of a highly selective sst_2 analog, octreotide, was beneficial in patients with DMARD refractory RA. (Paran, *et al.*, 2001). Thus, SRIF and its analogs have proven therapeutic benefits in preclinical and clinical RA studies, thereby providing a rationale for studying SRIF cellular actions in RA. Our current knowledge of SRIF actions in RA is limited and relevant intracellular signaling pathways remain unknown. However, a number of cellular targets and mechanisms exist for SRIF actions in RA.

A spectrum of immune cell-derived cytokines has been implicated in RA initiation and progression, although the pro-inflammatory cytokine, TNF-α, is thought to be a major contributor to prolonged RA pathogenesis (Feldmann, *et al.*, 2005). Clinical studies have demonstrated the utility of anti-TNF-α therapies (Feldmann, *et al.*; Genta, *et al.*, 2006). In the current study we address the possible therapeutic role of SRIF and SRIF analogs in modulating TNF-α effects on the synovium. The canonical pathway for TNF-α up-regulation of the mitogen-activated protein kinase (MAPK) family involves members of the extracellular regulated kinase (ERK), c-Jun terminal kinases (JNK), and p38 MAPK kinase families (Sweeney and Firestein, 2004). Of particular interest in the present study is the ERK1/2 signaling pathway in which TNF-α activates the RA synovium (Schett, *et al.*, 2000). ERK1/2 activation has been implicated in the control of cell proliferation, apoptosis, cytokine expression, and matrix metalloproteinase production (Firestein, 2004). ERK1/2 participates in a kinase cascade, and is active when phosphorylated by its upstream regulator, MEK1/2 (Figure 1). Phosphorylated ERK1/2 dimerizes and localizes to the cell nucleus where it serves to activate transcription, targeting transcription factors such as Elk-1 and c-myc, ultimately controlling cell cycle progression (Murga, *et al.*, 1999). Additionally, ERK1/2 activation is required for MMP-1, IL-6, IL-8, COX-2, and PGE gene expression in synovial fibroblasts, which may serve as pro-inflammatory mediators (Crofford, *et al.*, 2005; Pillinger, *et al.*, 2003; Neff, *et al.*, 2003). Previous studies have shown that the SRIF receptors control MAPK activity (reviewed in Vaysse, *et al.*, 2005), for example, the down regulation of protein kinases such as Raf(MAPKKK), MEK1/2 (MAPKK), and ERK1/2 (MAPK) is accomplished via

Figure 1. *Linear cascade of the ERK1/2 MAPK pathway.* Raf (MAPKKK) is a serine/threonine (S/T) kinase that activates MEK1/2 (MAPKK) by phosphorylating two specific serine residues (S217/221). MEK1/2 is a dual specificity kinase that activates ERK1/2 (MAPK) by phosphorylating both a tyrosine and a threonine residue (T202/Y204). ERK1/2 itself is a S/T kinase that targets both cytosolic and nuclear substrates. Upon phosphorylation, the two isoforms heterodimerize and enter the nucleus to phosphorylate and activate a host of transcription factors, thus regulating gene expression.

SRIF-induced phosphotyrosine phosphatases in sst, transfected Chinese hamster ovary (CHO) cells (Florio, *et al.*, 1999). However, the direct molecular connection between the sst_2 receptor and the ERK1/2 pathway has yet to be elucidated in RA synoviocytes.

Since RA synoviocytes express a molecular target for SRIF, and SRIF peptides and analogs have been proven to regulate ERK1/2 in other cell models, we studied the role of SRIF and an sst_2 subtype-specific analog, L-779,976, on cultured RA synoviocytes. Our data reveal that SRIF acts through the endogenously expressed sst_2 receptor to regulate RA synovial activated MAPK pathway proteins, possibly through the control of protein tyrosine phosphatase activity. The current study identifies a novel strategy to control ERK1/2-mediated inflammatory responses in the rheumatic synovium.

MATERIALS AND METHODS

Cell Culture

A commercial human fibroblast-like synoviocyte line (HFLS) was obtained from Cell Applications, Inc (CA). Additional primary synovial cell lines were isolated from surgical discard tissue obtained from the National Disease Research Interchange (NDRI; Philadelphia, PA). Protocols used in obtaining NDRI surgical discard specimens were reviewed by the Institutional Review Board of Seton Hall University and categorized as exempt by the IRB. Surgical discard tissue was minced, digested with 1mg/ml collagenase in serum-free media for 2 h at 37°C, and filter through a nylon mesh. The HFLS cells were cultured in T-75cm^2 flasks and 24-well cell culture dishes purchased from Corning (Corning, NY) in Dulbecco's modified Eagle's medium, DMEM, (with GlutaMAXTH, high glucose, 110mg L^{-1} sodium pyruvate and pyridoxine-HCL, Invitrogen; Carlsbad, CA) containing 100 U ml^{-1} of penicillin, 100μg ml^{-1} streptomycin, 250ng ml^{-1} of amphotericin B, and 10% fetal calf serum and incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air. The cell cultures were passaged at 75% confluence, and given 48 hours to recover prior to experimentation.

mRNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

Synovial mRNA was isolated using the MicroFast-Track™ 2.0 mRNA Isolation kit (Invitrogen Life Technologies, CA).

Isolated synovial mRNA was reverse transcribed and amplified via polymerase chain reaction with the Superscript One-Step RT-PCR system (Invitrogen Life Technologies, CA). Thirty cycles of PCR [94°C, 30 s; 58°, 30 s; 72°C, 2 m] were performed following a reverse transcriptase step at 50° for 30 m, and a final elongation step at 72°C for 5 m. The primer sets used are: sst_1-f = gcaacatgctcatgcc, sst_1-r = gcgttatccgtccagg, sst_2-f = agagccgtactatgacctga, sst_2-r = agccactcggattccagag,(Invitrogen Life Technologies, CA) sst_3-f = tcatctgcctctgctacctgc, sst_3-r = gagcccaaagaaggcaggct, sst_4-f= catggtcgctatccagtgca, sst_4-r = gtgagacagaagacgctggtgaacat, sst_5-f = gctcctggtgttcgcggacgt, sst_5-r = gaggatgaccacgaagaagtagagg (Integrated DNA Technologies, Coralville, IA). The final reaction volume was 50µl in 1X Reaction Mix buffer containing RT/Platinum Taq Mix, 10nM each forward and reverse primers, and template mRNA. Thermal cycling was performed using a Perkin Elmer Thermal Cycler 9600 (Perkin Elmer, MA). Sst full-length cDNA clones were used as positive controls for each primer pair.

RT-PCR products were resolved on 1.25% agarose gel in 1x TAE buffer (Invitrogen Life Technologies, CA) and stained with ethidium bromide (10µg/ml). DNA products were visualized using a UV transilluminator (UVP, Inc., CA), and photo images were obtained with both a 35 mm Polaroid camera and digital images were captured with AlphaImager 3.3D (San Leandro, CA) computer software.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Synoviocytes were cultured in 24-well plates with complete media for 48 h and then starved in serum-free media for 24 h in order to establish basal cell activity. The cultured cells were then incubated in the presence or absence of 100nM SRIF-14 and L-779,976 in serum-free media for 15 min at 37°C. SRIF-14 was purchased from Peninsula Labs (Belmont, CA). L-779,976 was provided through a Materials Transfer Agreement between Seton Hall University and Merck & Co, Inc (Rahway, NJ). Following treatment, synoviocytes were lysed using 1X NuPAGE LDS sample buffer (Invitrogen Life Technologies, CA). Cell lysates were sonicated and heated to 70°C for 10 min, resolved on NuPAGE 10% Bis-Tris gels (Invitrogen Life Technologies, CA), electroblotted onto polyvinyldifluoride (PVDF) membranes and treated for 45 min in a blocking buffer containing 5% (w/v) non-fat dehydrated milk. Phospho-ERK1/2 was then immunodetected with a 1:1000 dilution of affinity purified phospho-ERK1/2 antisera (Cell Signaling Technology, MA). The membrane was then incubated for 1 hour in a 1:2000 dilution of a horseradish peroxidase-conjugated secondary antibody and the retained immunoreactivity detected using ECL chemiluminescence, according to the manufacturer's instructions (Amersham, NJ). The resulting autoradiograph was scanned and densitometric analysis was performed with NIH ImageJ and quantified using GraphPad Prism 4.0 (GraphPad Software, CA).

Protein tyrosine phosphatase assay

Synovial fibroblast cultures were incubated in the presence or absence of 100nM SRIF-14 (Peninsula Labs, Belmont, CA) for 15 min. Cell cultures were washed in PBS and then lysed in a 50mM HEPES buffer containing 0.5% Triton X-100, 10% glycerol (GibcoBRL), 2.5μM pepstatin A, 12.5μM leupeptin, 12.5μM aprotinin, 12.5μM bacitracin, and 200nM polymethylsulfonyl fluoride. Lysates were then centrifuged at 13,500 x g for 15 min at 6°C and the supernatant retained.

Protein phosphatase assays were performed in a 96 well plate (Corning, Corning, NY) as described (Badway *et al.*, 2004). Briefly, synovial cell membranes were incubated with 50μM of the fluorescent phosphatase substrate, 6,8-difluoro-4-methylumberiferyll phosphate (DiF-MUP; Molecular Probes, OR; Gee *et al.*, 1999) up to 30 min at 37°C with intermittent mixing in a CytoFluor Series 4000 fluorescent plate reader (Applied Biosystems, CA). Fluorescence was detected using 360nm excitation and 460nm emission filters. Replicate parallel assays were conducted in the presence of 10μM Na_3VO_4, an inhibitor of tyrosine phosphatase activity or 10 mM phosphatase inhibitor cocktail (Sigma, St Louis, MO), a commercially available inhibitor cocktail for serine/threonine phosphatases. The resulting data were analyzed with GraphPad Prism 4.0 as described (Badway *et al.*, 2004).

Fluorescence confocal scanning microscopy

Synoviocytes were cultured on 4-well, 1.8 cm^2/well glass chamber culture slides (LabTek) with complete media (DMEM) for 48 h, then cultured in serum-free media for 24 h, and then incubated in the presence or absence of 100nM SRIF-14 plus or minus 10ng/ml TNFα for 15 min at 37°C. The cell monolayers were then fixed in 4% paraformaldehyde, rinsed extensively in PBS and then incubated with phospho-ERK1/2 antisera (1:1000) in 1% normal goat serum (Vector Laboratories, Burlingame, CA). Fluorescein (Vector Laboratories, CA) was used as the secondary detection antibody (fluorescein) and the incubation was carried out in 1% normal goat serum. After extensive washing, the slides were prepared in Vectashield (Vector Laboratories, CA). Confocal fluorescent microscopy and digital image capture were performed with an Olympus Fluoview™ FV1000 Confocal Microscope (Olympus, USA) and Olympus Fluoview FV10-ASW Version 1.3a software (Olympus, USA); the fluorescein conjugated immunoreactivity was excited at 485nm and the emitted at 530nm and was digitally captured.

RESULTS

Identification of sst_2 on synovial fibroblasts via RT-PCR

RT-PCR was performed, using SRIF receptor subtype specific oligonucleotide primer pairs to establish a potential target for SRIF interaction. Figure 2A demonstrates the ability of five SRIF receptor subtype-specific oligonucleotide primer pairs to amplify cloned cDNAs of sst_{1-5}. Figures 2B-2D summarizes our findings on several synovial cell cultures. We demonstrate that sst_2 is present and appears as a 600 base pair amplified product in three different RA synovial tissues, while sst_s 1, 3, 4, and 5 are absent. Our data corresponds with literature identifying sst_2 on RA synovium (ten Bokum, *et al.*, 1999; Lu, *et al.*, 2001), however differs from a group who have reported the presence of sst_1 in addition to sst_2 in synovial fibroblasts (Takeba, *et al.*, 1997).

Figure 2. *RT-PCR reveals sst_2 on synovial fibroblasts.* **A.** PCR amplification of human cDNA clones for sst_{1-5} was performed to ensure that the SRIF subtype specific oligonucleotide primer pairs were capable of amplifying their target sequence. **B.** RT-PCR amplification of total RA synovial mRNA (Cell Applications). **C-D.** Two different RA synovial surgical tissue samples amplified with SRIF receptor subtype specific oligonucleotide primer pairs (NDRI).

Western Blot detection of phosphorylated ERK1/2 in RA synoviocytes

MAPKs (mitogen activated protein kinases) regulate gene expression, cell proliferation, cell survival and death, and cell motility in mammalian models (Chang and Karin, 2001). ERK1/2 (extracellular regulated kinases -1 and -2) are examples of these kinases that are expressed and activated in the RA synovium (Schett, *et al.*, 2000). We assessed the activation of ERK1/2 with phospho-specific antiserum via Western blotting experiments in response to SRIF and SRIF analog treatment.

We reveal the ability of 100nM SRIF to reduce phospho-ERK1/2 by 34 ± 4% from basal levels in RA synoviocytes (Figure 3, *$p<0.05$ control versus SRIF treated). Furthermore, the sst_2 selective SRIF analog, L-779,976, reduced phospho-ERK1/2 by 46±7% from basal levels in RA synoviocytes (Figure 3, **$p<0.01$ control versus L-779,976 treated).

Another MAPK protein, p38, has been indicated as a possible target for RA therapy (Peifer, *et al.*, 2006). We saw no difference in active p38 levels between control and SRIF treated RA synoviocytes via Western blotting (data not shown). Furthermore, to ensure the effects of SRIF are mediated through the ERK1/2 pathway, we used phospho-specific Raf and MEK1/2 antisera to assess the levels of activated upstream regulators of ERK1/2. We found that acute SRIF reduced phospho-Raf by 13±5% and phospho-MEK1/2 by 48±5% from basal levels in RA synoviocytes (Figure 4, $p>0.05$, and **$p<0.01$ control versus SRIF treated for phospho- Raf, and MEK1/2, respectively).

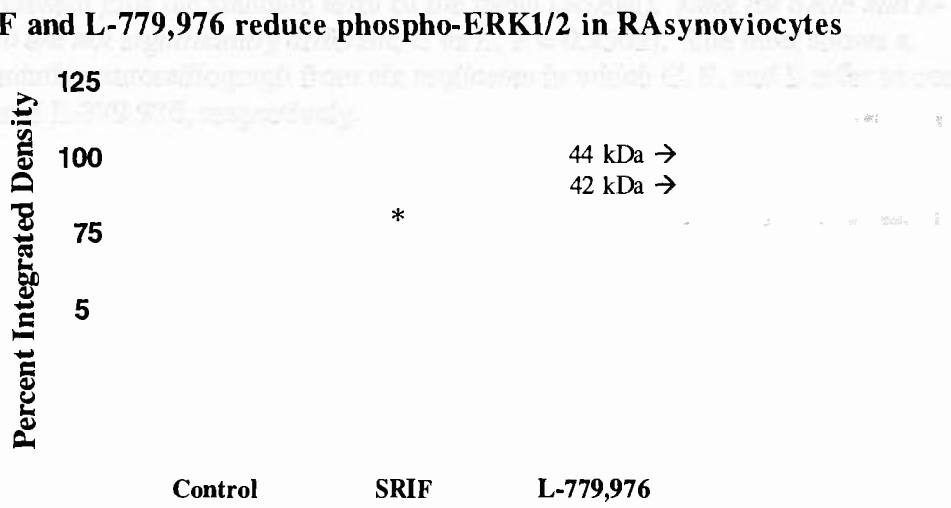


Figure 3. *SRIF-14 and L-779,976 reduce phosphorylated ERK1/2 from basal levels in RA HFLS.* Parallel cultures of RA HFLS cells were cultured in serum-free basal medium for 24 h and then incubated in the presence or absence of 100 nM SRIF-14 or L-779,976 for 15 min at 37°C. Cell lysates were prepared and resolved on NuPAGE 10% Bis-Tris gels, electroblotted, and incubated overnight with a 1:1000 dilution of an antiphospho-ERK1/2 antibody (Cell Signaling, Beverly, MA). Immunoreactivity was detected using a horseradish peroxidase-conjugated anti-rabbit second antibody in conjunction with chemiluminescence. Autoradiographs were quantified using NIH ImageJ software and data the were analyzed using Graphpad Prism 4.0. SRIF and L-779,976 data were normalized and analyzed compared to control; Student t-test shows these results are significantly different: C vs S, $P = 0.0011$, C vs L, $P = 0.0059$, error bars represent plus the standard error of the mean (+SEM). Data for SRIF and L-779,976 are not significantly different, C vs L, $P = 0.2562$). The inset shows a representative autoradiograph from six replicates in which C, S, and L refer to control, SRIF, and L-779,976, respectively.

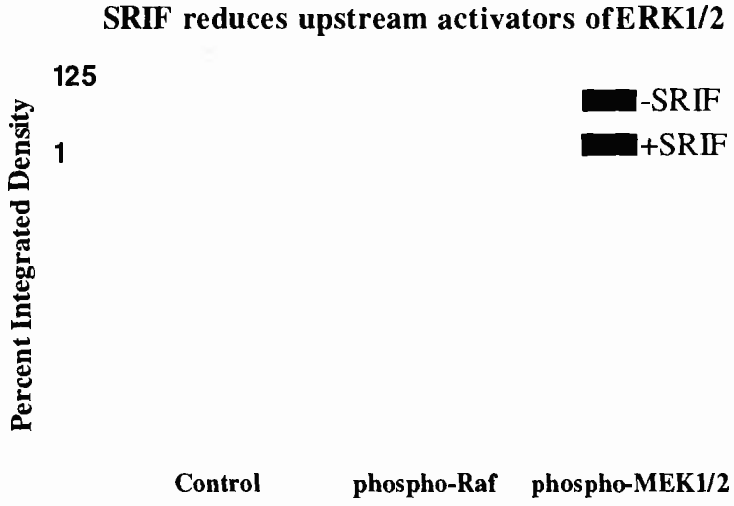


Figure 4. *SRIF-14 reduces phosphorylated Raf and MEK1/2 from basal levels in RA HFLS.* Parallel cultures of RA HFLS cells were cultured in serum-free basal medium for 24 h and then incubated in the presence or absence of 100 nM SRIF-14 for 15 min at 37°C. Cell lysates were immunoblotted with phospho-specific Raf and MEK1/2 antisera similarly to the protocol outlined in Figure 3. Autoradiographs were quantified using NIH ImageJ software and data was analyzed using Graphpad Prism 4.0. SRIF data were normalized and analyzed compared to control. Student t-test shows phospho-Raf data are not significantly different from control: C vs S, P = 0.1202; the error bars represent +SEM. Phospho-MEK1/2 are statistically significant: C vs S, P = 0.0075; the error bars represent +SEM. This experiment was performed in duplicate.

Determination of membrane phosphoprotein tyrosine phosphatase activity

SRIF controls several intracellular phosphotyrosine phosphatases, thereby providing a basis for SRIF's well documented effects on cell proliferation (Patel, 1999). We investigated the effects of SRIF and L-779,976 on the RA synoviocyte tyrosine phosphatase activity. We utilized DiF-MUP as a synthetic fluorescent phosphatase substrate, in conjunction with sodium vanadate which selectively inhibits protein tyrosine phosphatase activity. In particular, we investigated phosphatase activity in both the membrane and in soluble lysates of RA synoviocytes. SRIF increased phosphatase activity in RA synoviocytes by 3-fold from basal levels (*$p<0.05$, C vs S) and this affect was abolished by sodium vanadate (Figure 5). SRIF did not affect soluble phosphatase activity (data not shown).

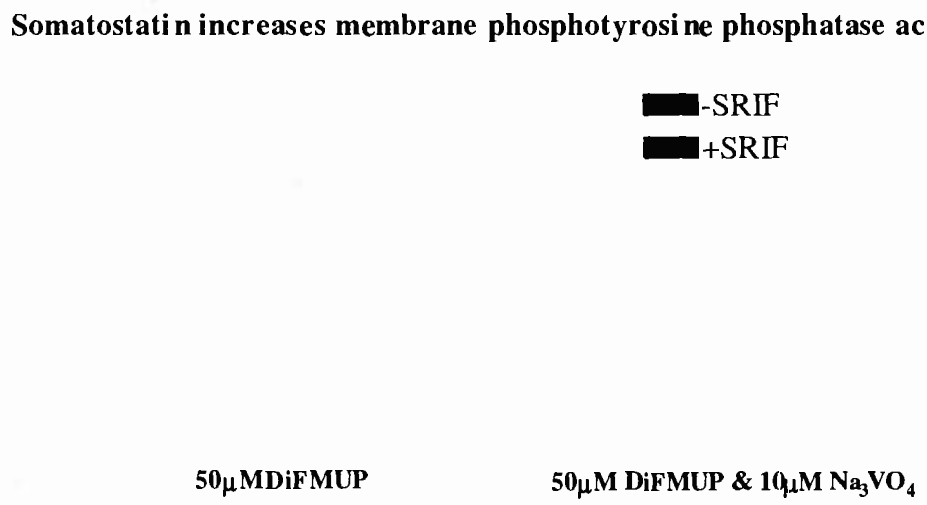
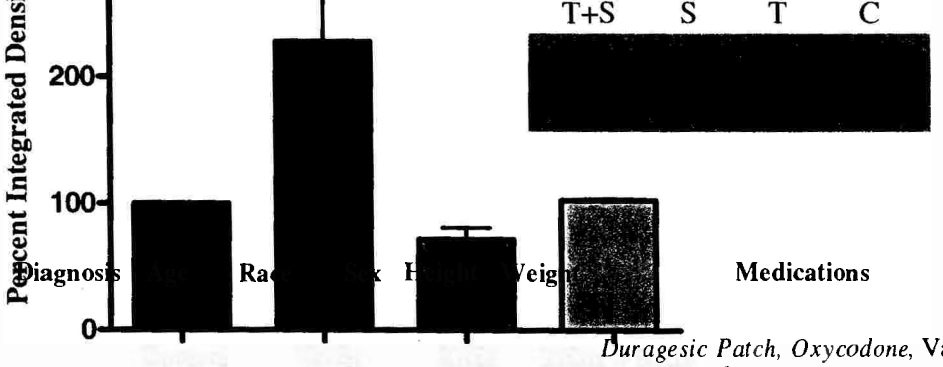


Figure 5. ***SRIF activates phosphatase activity in RA HFLS.*** RA synoviocytes (Cell Applications, Inc., CA) were grown to 75% confluence on T-75 cm- flasks and were incubated in the absence or presence of 100 nM SRIF-14, and 10 μM Na_3VO_4. Membranes were prepared in 50 mM HEPES, 0.5% Triton X-100, 10% glycerol, 2.5 μM pepstatin A, 12.5 μM leupeptin, 12.5 μM aprotinin, 12.5 μM bacitracin and 200 nM PMSF. The suspension was incubated with 50 μM fluorescent phosphatase substrate (DiF-MUP; Gee *et al.*, 1999) for 10 min at 37°C with intermittent mixing in a CytoFluor 4000 fluorescent plate reader (Applied Biosystems). Student's T-test shows statististical difference, P = 0.0103, Control vs SRIF, 50μM DiFMUP.

Western Blot detection of phosphorylated ERK1/2 in synovial tissue samples

To further our study, we investigated the levels of ERK1/2 via immunoblotting in tissues received from NDRI, presumed to be from RA patients. The RA synovial tissue samples were selected for study based on the patients' medical history (Table 1). The patient and primary cell lines created from these RA tissue samples were identified arbitrarily (RA2 & RA3). Prednisone, a glucocorticoid, and the nonsteriodal anti-inflammatory drug (NSAID), Bextra were used to treat patient RA2, while Diclofinac (NSAID) and a disease modifying anti-rheumatic drug (DMARD) Azulfadine were used to treat patient RA3. In other RA tissues (RA1 and RA4), it was unclear that these patients were in fact suffering from the disease because they were not prescribed typical RA medications (information not shown).

We investigated SRIF modulation of phospho-ERK1/2 in the RA3 synovial cell line in the absence or presence of TNFα. Previously, we have shown that SRIF decreased phospho-ERK1/2 by 34±4% in a commercial RA cell line (Figure 3). Again, we demonstrate SRIF's ability to reduce phospho-ERK1/2 by 28±9% in RA3 synovial monolayers (Figure 6, $p>0.05$ control versus SRIF treated). TNFα increased phospho-ERK1/2 by 128±53% in RA3 synoviocytes (Figure 6, $p>0.05$ control versus TNFα treated). Of particular interest was the ability of 100nM SRIF to reduce TNFα-stimulated phospho-ERK1/2 to basal levels (Figure 6, $p>0.05$ TNFα treated versus TNFα + SRIF treated).



Cell Line	Diagnosis	Age	Race	Sex	Height	Weight	Medications
RA2	RA	67	Caucasian	F	5' 2"	123 lbs	*Duragesic Patch, Oxycodone,* Valtrex, Levoxyl, **Prednisone**, **Bextra**, Colestid, Folic Acid
RA3	RA	39	Caucasian	F	5' 2"	117 lbs	Tegretol, Klonapine, Prozac, **Azulfadine, Diclofinac,** Prilosec, Topamax, Replax

Table 1. *RA synovial tissue donor information.* Patient history appears in tabulated form; medical history and pharmacology were considered when selecting synovial tissues to study. Medications in bold and underlined font are utilized in treatment of patients with rheumatoid arthritis. The medications in italics may have been used as analgesics to control pain associated with RA. Because the donor retains anonymity, a medical consult was not possible.

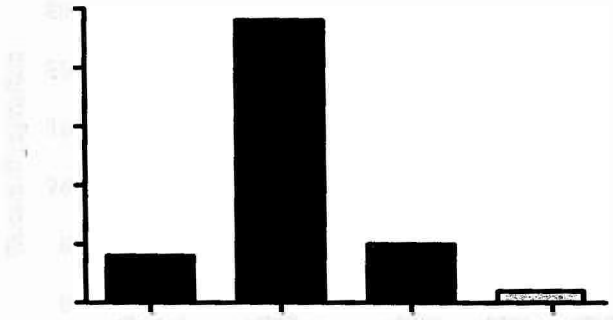
SRIF & TNFα effects on phospho-ERK1/2 in RA synovial tissue

Control **TNFα** **SRIF** **TNFα + SRIF**

Figure 6. *SRIF-14 reduces TNFα-induced ERK1/2 phosphorylation in RA synoviocytes derived from tissue donors.* Monolayer cultures from the RA3 tissue sample of RA synoviocytes were immunoblotted with phospho-ERK1/2 antiserum as outlined in Figure 3. Autoradiographs were quantified using NIH ImageJ software and data was analyzed using Graphpad Prism 4.0. Data were normalized and analyzed compared to control or TNFα treated; Student t-test shows that these results are not significantly different: C vs T, P = 0.0953; C vs S, P = 0.0949; C vs T+S, P = 0.1891; T vs T+S, P = 0.2809, error bars represent +SEM. The inset shows a representative autoradiograph from three replicates in which C, T, S, and T+S refer to control, TNFα, SRIF, and TNFα+SRIF, respectively.

Fluorescence detection of phosphorylated ERK1/2 in synovial tissue samples

Confocal fluorescence microscopy was performed to visualize the location of phosphorylated ERK1/2 in the RA3 synoviocyte. Cells were treated in the absence or presence of SRIF and TNF-α for 15 m. Under control conditions, phospho-ERK1/2 did not accumulate in the nucleus (Figure 7A). However, acute TNF-α treatment caused activation and movement of ERK1/2 into the nuclei of approximately 24% of the cell population. (Figure 7B, 7E). Acute SRIF treatment also promotes the recruitment of phosphorylated ERK1/2 into the nuclei of a small population of cells (Figure 7C, 7E). However, these cells do not appear to hold the same morphology as the synovial fibroblasts. We have not yet identified the phenotype of these cells. Upon acute co-treatment of SRIF and TNFα, phospho-ERK1/2 does not accumulate in the nuclei of these cells (Figure 7D). These results indicate that the tissue population is heterogeneous, and suggest only a portion of the population are transformed synoviocytes.



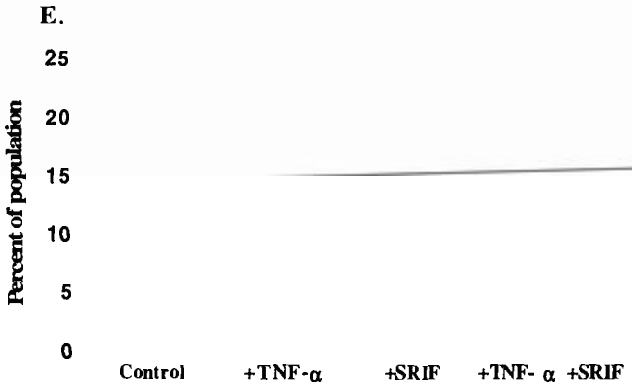


Figure 7. *Fluorescence confocal scanning microscopy of synovial tissue.* Monolayer cultures of synoviocytes from the RA3 tissue donor were treated in the absence or presence of SRIF (100nM) and TNFα (10ng/ml): **A**. Control, **B**. TNFα, **C**. SRIF, **D**. TNFα + SRIF. After treatments, the cells were fixed in 4% paraformaldehyde, probed with phospho-ERK1/2 antiserum, and detected with fluorescein secondary antibody, both in 1% normal goat serum. Microscopy and digital photography were performed with an Olympus FluoviewTM FV1000 Confocal Microscope, 485nm excitation, 530nm emission. **E**. Representative bar graph showing the percentage of cells with activated ERK1/2 in the nucleus.

DISCUSSION

Rheumatoid Arthritis (RA) is a debilitating autoimmune disease in which affected individuals suffer from decreased mobility due to bone and cartilage destruction. The initial immune trigger is unknown, however it is believed that synovial dysfunction plays a critical role in disease progression, as well as RA associated morbidity and mortality (Zvaifler and Firestein, 1994; Takeba, *et al.*, 1997). Understanding the underlying signal transduction of the RA synoviocyte might prove critical in developing novel therapeutic treatments to retard the progression of the chronic disease.

Mitogen activated protein kinases (MAPKs) are highly conserved intracellular signaling proteins that exist in several distinct families in vertebrates (Chang and Karin, 2001). The MAPKs regulate a spectrum of cellular activity, including cell survival, proliferation and intercellular signaling. In the RA synoviocyte, MAPKs have been implicated in pro-inflammatory cytokine production and metalloproteinase production, cellular products that promote synovial dysfunction and joint destruction (Sweeney and Firestein, 2004). Since the RA synoviocyte is an active participant in the RA process, synovial targets that could mitigate or silence these inflammatory responses could prove useful in RA treatment. Several reports have recently appeared indicating that the ubiquitous neuropeptide, SRIF, might play a role in controlling RA synovial responses.

(reviewed in Paran and Paran, 2003). However, to date, no signal transduction mechanism for SRIF action in the synoviocyte has been proposed.

In our initial studies we observed that the native peptide SRIF and the sst_2 subtype peptidomimetic, L-779,976, suppressed basal ERK1/2 phosphorylation, suggesting that in the RA cells examined, there was an elevation of basal ERK1/2 phosphorylation. This elevation of basal ERK1/2 activity could be explained by the constant exposure of the synovium to pro-inflammatory cytokines in the RA joint. In several studies, SRIF has been implicated as a regulator of MAPKs, specifically ERK1/2. In other *in vitro* disease models, several protein tyrosine phosphatases have been proposed to mediate the SRIF effect (Badway and Blake, 2005; Vaysse *et al.*, 2005). Interestingly, SRIF modulation of the ERK1/2 MAPK pathway is both receptor subtype and cell specific, demonstrating the need to delineate among the receptor subtypes present in specific cell populations (Blake, *et al.*, 2004; Lahlou, *et al.*, 2004).

In this study we have identified, via RT-PCR, the SRIF receptor subtype 2 (sst_2) in cell cultures derived from human synovial tissue. In contrast with an earlier study, we were unable to detect sst_1 mRNA which had been previously reported to exist along with sst_2 in the synovium (Takeba, *et al.*, 1997). Although both studies used synovium derived from the knee, Takeba and colleagues (Takeba, *et al.*, 1997) also included synovial membrane from other body regions including the wrist. Since SRIF receptor expression is anatomically heterogeneous, the inclusion of the wrist synovium may have been responsible for the presence of sst_1 mRNA. Indeed, our control PCR amplifications indicated that the oligonucleotide pairs used for sst_1 amplification in this study were able

to amplify the human sst_1 cDNA and the correct PCR product size was obtained (Figure 2A), suggesting that any sst_1 mRNA present would have been detected in our protocol.

The synovial fibroblasts are known targets of pro-inflammatory cytokines, and upon activation may participate in the initiation and development of joint destruction (Davis, 2003). TNF-α is a critical cytokine in the arthritic pro-inflammatory response and is considered a major therapeutic target in RA (Reimold, 2002). Takeba and colleagues reported that TNF-α treatment up-regulated IL-6 and IL-8 cytokine expression and MMP production, both integral to RA development and pathogenesis (Takeba, *et al.*, 1997). Of considerable interest, was also the observation that SRIF treatment was able to suppress these TNF-α mediated increases, which included decreasing cellular mRNA levels for IL-6, IL-8, MMP-1, MMP-2 and MMP-9 (Takeba, *et al.*, 1997). Takeba and colleagues (Takeba, *et al.*, 1997) hypothesized that SRIF inhibition of the transcription factor, cyclic AMP response element binding protein (CREB), was responsible for this downregulation of synovial interleukin and MMP mRNA. The SRIF suppression of cyclic AMP production by adenylyl cyclase, and the corresponding inhibition of cyclic AMP-dependent protein kinase (PKA) are hallmarks of SRIF action in all cells studied to date (Blake, *et al.*, 2004). A corresponding decrease in CREB activation and migration to the cell nucleus would then be predicted, if SRIF were controlling the synovial PKA signaling pathway. Indeed, a modest reduction in CREB was observed in the synovial cells that were co-treated with TNF-α and SRIF, suggesting that SRIF may decrease the synovial intracellular cAMP levels. However, it is hard to reconcile the modest decrease in CREB nuclear transport observed by Takeba and colleagues (Takeba, *et al.*, 1997) with

the almost complete ablation of the synovial IL-6, IL-8, and MMP mRNA that was also noted. Therefore, an additional intracellular signaling pathway must be controlled by SRIF in the RA synoviocytes. Our current results support this notion.

In our study we utilize two SRIF ligands, SRIF-14, the most prevalent endogenous form of SRIF, and L-779,976, an sst_2 receptor subtype selective, nonpeptidyl, SRIF analog, to reveal SRIF modulation of ERK1/2 via an sst_2 mediated pathway in RA synoviocytes. More specifically, SRIF reduced activated ERK1/2 in these cells by 34 ± 4% while the synthetic analog, L-779,976, reduced ERK1/2 phosphorylation by 46±7% (Figure 3). There was no statistical difference between SRIF and L-779,976 treatments (Figure 3, $p>0.05$ S vs L). The phosphorylation state of ERK1/2 parallels functional activity of this protein kinase family, with increasing phosphorylation correlating with increased ERK1/2 activity (Chang and Karin, 2002). Reducing active ERK1/2 levels in RA synoviocytes could suppress synovial hypertrophy, hyperplasia, and cytokine secretion, all of which are characteristic of a transformed synoviocyte (Davis, 2003). Our results (Figure 3) clearly indicate that SRIF treatment of the RA synoviocytes decreases the phosphorylation status of ERK1/2 in these cells, suggesting an additional mechanism by which SRIF could suppress IL-6, IL-8, and MMP production in fibroblasts (Takeba, *et al.*, 1997)

It is not fully understood how SRIF receptor activation ends with ERK1/2 in the nucleus. Tyrosine phosphorylation is a rare event in the cell, and could indicate a novel method by which to control ERK1/2 activation in the synoviocyte. ERK1/2 activation involves the reversible phosphorylation of a tyrosine and threonine, a process which is

reversed by a family of dual specificity protein phosphatases (Farooq and Zhou, 2004). To date, SRIF activity has not been shown to directly activate any of the known dual specificity protein phosphatases that regulate ERK1/2 activity. Alternatively, SRIF has been shown to control ERK1/2 through the upregulation of protein tyrosine phosphatase (PTP) activity, and the subsequent dephosphorylation and inhibition of the upstream activating kinases in the ERK1/2 pathway (Vaysee, *et al.*, 2005). At this time, the identity of this tyrosine phosphatase, controlling the ERK1/2 pathway, is not known. Correspondingly, we observed an SRIF-induced suppression of Raf and MEK1/2 phosphorylation, two upstream kinases regulators of ERK1/2. Thus, we investigated whether SRIF could modulate tyrosine phosphatase activity within the RA synoviocyte. We used a fluorescent surrogate substrate for phosphorylated tyrosine, 6,8-difluoro-4-methylumberiferyll phosphate (DiF-MUP), which when used in conjunction with the tyrosine phosphatase inhibitor, sodium vanadate, provides a robust and reliable measure of protein tyrosine phosphatase activity (Badway, *et al.*, 2004). We observed that in our DiF-MUP assay, SRIF increased PTP activity in the membranes derived from SRIF-14 treated cells by approximately 3-fold (Figure 4). Co-incubation of the cell membranes with the tyrosine phosphatase inhibitor, sodium vanadate, completely abolished this activity, suggesting that tyrosine phosphatase activity was under SRIF control in the synoviocytes. It is unclear where these PTPs intercede the ERK1/2 pathway in RA synoviocytes, however it can be hypothesized that membrane-associated proteins are the substrate of preference since SRIF did not affect soluble PTP activity in these RA synoviocytes (data not shown). The inhibition of membrane associated tyrosine

phosphatase activity also suggests that the dual-specificity tyrosine phosphatases are unlikely to be the target of SRIF control in the RA synoviocytes, since the activity of the dual specificity phosphatases appears to be largely restricted to the cytosolic and nuclear compartments (Farooq and Zhou, 2004).

Utilizing tissue procured from the National Disease Research Interchange (NDRI), we investigated activated ERK1/2 levels in synoviocytes (RA3) cultured from an RA patient tissue sample. We found that this tissue responded to SRIF treatment comparatively to the commercial RA tissue in our initial studies. In fact, SRIF reduced levels of phosphorylated ERK1/2 by 28±9% in these cells from basal levels as determined by western blotting (Figure 6). Our data also support findings that TNF-α activates ERK1/2 in the RA synovium (Figure 6; Lacey, *et al.*, 2002). In intestinal epithelial cells, SRIF reduced both basal and TNF-α-induced secretion of IL-8 and IL-1β, demonstrating its anti-inflammatory effect in this *in vitro* model (Chowers, *et al.*, 2000; reviewed in Paran and Paran, 2003). We reveal that SRIF inhibits TNF-α-stimulated upregulation of ERK1/2 in RA synoviocytes, suggesting SRIF's potential modulation of cytokine and pro-inflammatory mediator release via TNF-α attenuation.

To further investigate and verify the previous immunoblotting experiments, we used fluorescence confocal scanning microscopy to visualize ERK1/2 localization within the RA3 synovial fibroblasts. Our results reveal that TNF-α activates ERK1/2 in a sample of the synovial population (Figure 7B). It appears that SRIF also activates a minute subset of the population, however these cells have different morphological features as compared to synovial fibroblasts (Figure 7C). Co-treatment of SRIF and

TNF-α had no effect on the nuclear localization of ERK1/2, confirming SRIF's ability to inhibit TNFα-stimulated ERK1/2 activation (Figure 7D). Interestingly, the entire cell population does not respond to TNFα stimulation, suggesting that this tissue is in fact a heterogeneous population of cells perhaps composed of diseased and non-diseased synoviocytes. We suggest that these tissues are not advanced or refractory RA tissues; our skepticism lies in the medical history of the patients. Since the early 90s, the gold-standard for RA treatment has been methotrexate, which was not used to treat any of our four RA tissue donors (Table 1). Although some patients are not responsive to methotrexate, it is has been the dominant choice of DMARDs because of its low-cost, effectiveness in retarding the disease, and tolerability (Choi, *et al.*, 2000).

CONCLUSIONS

In this study, we reveal a regulatory role of SRIF in a RA synoviocyte *in vitro* model, identifying a target, the sst_2 receptor for SRIF intervention in the human synovial fibroblast. We have demonstrated that SRIF decreases ERK1/2 levels in RA synoviocytes, suggesting that basal ERK1/2 is upregulated in this tissue. Additionally, we have shown that the inhibition of ERK1/2 in RA synoviocytes may be associated with SRIF-mediated up-regulation of protein tyrosine phosphatases. In tissue cultured from RA patients, we found that SRIF suppressed both basal and TNF-α-stimulated ERK1/2 levels, indicating SRIF's ability to attenuate TNF-α effects on RA synoviocytes. Further studies delineating the protein-protein interactions, under SRIF control, of the ERK1/2 MAPK pathway could provide new targets for RA therapy

Future studies utilizing synovial tissue from true refractory RA patients could provide a more homogeneous cell population to study SRIF's effects on ERK1/2 activation. Additionally, treating normal and non-refractory RA synoviocytes with a cocktail of cytokines and chemokines in attempt to transform these cells, could provide insight into the differentiation process of an RA synoviocyte. This information could aid in developing novel therapies that target cytokine-mediated events in RA synoviocytes.

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