In vitro chondrogenesis

The role of microRNAs during differentiation and dedifferentiation

Doctoral thesis

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Overview of the thesis

The overall aim of this thesis was to investigate the role of microRNAs (miRNAs) during dedifferentiation and chondrogenic differentiation of articular chondrocytes (ACs) and mesenchymal stem cells (MSCs), respectively. miRNAs are small RNA molecules that regulate gene expression in a sequence-specific manner by binding to complementary mRNA sequences often leading to degradation of the mRNA or translational repression. In other situations, miRNAs may enhance gene expression. When this thesis was begun, there were relatively few studies on cartilage biology that involved miRNAs, and no studies had investigated global miRNA changes during dedifferentiation of human ACs or chondrogenic differentiation of human MSCs.

As miRNAs are regarded to be important regulators of gene expression in most cell types, we decided to perform a global miRNA analysis of both ACs and MSCs to identify miRNAs that were important for cartilage biology and to investigate their function and identify their targets. In paper I and II we identified miR-140 as an important miRNA during dedifferentiation of ACs and chondrogenic differentiation of MSCs. In vivo studies have previously identified miR-140 as an important miRNA for cartilage development and homeostasis, but the mechanism has not been fully understood. In paper II, we show that miR-140 positively regulated the master transcription factor of chondrogenesis SOX9 and the proteoglycan ACAN at the post-transcriptional level. The results provided here may explain the profound effect of miR-140 in cartilage biology.

In paper III, we performed gain- and loss-of-function studies using transient transfection of small synthetic double-stranded microRNA mimics (smiRs) and inhibitors. Surprisingly, transfection of smiR-145, but not any of the other synthetic molecules tested, lead to a strong immune response in both MSCs and ACs. The immune response was not a result of smiR-145 regulating its target genes, but a result of the cell responding to smiR-145

as a foreign molecule. Such immune responses complicate the interpretation of the results. In paper III, we investigated this phenomenon and identified the receptor responsible for the immune response. Induction of the immune response required liposome delivery of smiR-145, as no immune gene changes were observed after delivery of smiR-145 directly into the cytosol using electroporation. This insight is important for researchers to avoid unexpected results from transient transfection experiments in vitro and unwanted immune responses following the use of liposome transfection reagents in vivo.

List of papers included

Paper I

Human primary articular chondrocytes, chondroblasts-like cells, and dedifferentiated chondrocytes: differences in gene, microRNA, and protein expression and phenotype.

Karlsen TA, Shahdadfar A, Brinchmann JE. Tissue Eng Part C Methods. 2011 Feb;17(2):219-27.

Paper II

microRNA-140 regulates chondrogenic differentiation of human mesenchymal stem cells by post-transcriptional enhancement of chondrogenic molecules.

Karlsen TA*, Jakobsen RB*, Mikkelsen TS, Brinchmann JE. Submitted.

*Joint first authors

Paper III

Liposome delivery of microRNA-145 to mesenchymal stem cells leads to immunological off-target effects mediated by RIG-I.

Karlsen TA, Brinchmann JE. Molecular Therapy, accepted February 2013.

Abbreviations

ACAN	aggrecan
ACs	articular chondrocytes
ACI	autologous chondrocyte implantation
ALPL	alkaline phosphatase
AQ	absolute quantification
ASCs	adult stem cells
BMP	bone morphogenetic protein
CACI	collagen-covered ACI
CZ	calcified zone
DZ	deep zone
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
ESCs	embryonic stem cells
FACS	fluorescence activated cell sorting
FBS	foetal bovine serum
FGF	fibroblast growth factor
GAGs	glycosaminoglycans
HA	hyaluronic acid
HCV	hepatitis C virus
hPLP	human platelet lysate-rich plasma
HSCs	hematopoietic stem cells

ICM	inner cell mass
IF	immunofluorescence
IHC	immunohistochemistry
IHH	Indian hedgehog
IL	interleukin
iPSCs	induced pluripotent stem cells
LGP2	laboratory of genetics and physiology 2
LIF	leukaemia inhibitory factor
MACI	matrix-induced ACI
MDA5	melanoma associated gene 5
miRNAs	microRNAs
MMP	matrix metallopeptidase
MOI	multiplicity of infection
MSCs	mesenchymal stem cells
MYD88	myeloid differentiation primary response 88
MZ	middle zone
OA	osteoarthritis
PAMPs	pathogen-associated molecular patterns
ppp	triphosphate
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PRRs	pattern recognition receptors
PTHrP	parathyroid hormone-like peptide

RIG-I	retinoic acid-inducible gene-I
RLRs	retinoic acid-inducible gene-I-like receptors
RQ	relative quantification
RT	reverse transcription
RT-qPCR	reverse transcription - quantitative real-time polymerase chain reaction
RUNX2	runt-related transcription factor 2
siRNAs	small interfering RNAs
SZ	superficial zone
TGF-β	transforming growth factor beta
TLR	toll-like receptor
TRIF	TIR-domain-containing adapter-inducing interferon-β
VEGF	vascular endothelial growth factor
WNT	wingless

Introduction

Cartilage

Cartilage is a connective tissue found in different parts of the body, such as the ear, intervertebral discs and in the joints. There are three different types of cartilage: elastic cartilage, fibrocartilage and hyaline cartilage. All three types consist of water and an extracellular matrix (ECM) that gives the cartilage its unique properties. However, the three types of cartilage consist of different types and amounts of ECM molecules that are organised in different ways, giving each type of cartilage unique properties to fulfil its function in different parts of the body. In this thesis, the focus is on articular cartilage, the hyaline cartilage that covers the articular surfaces of bones.

Articular cartilage

The main function of articular cartilage is to allow for smooth movement of our bones against each other and to absorb and transmit the mechanical load resulting from daily activities, such as walking or sports activities. Articular cartilage is a highly specialised tissue. It has no blood supply, it is not innervated, and it obtains nutrients mainly by diffusion from the synovial fluid inside the joint [(1)]. Articular cartilage is approximately 2-2.5 mm thick (with anatomical and topographical variations) [(2,3)], consisting mainly of water, collagens, proteoglycans, glycosaminoglycans (GAGs) and smaller amounts of glycoproteins and non-collagenous molecules [(4)]. The cells in articular cartilage are called articular chondrocytes (ACs), and they make up approximately 2% of the tissue volume [(3)].

The scope of this thesis does not allow a detailed, complete description of all the molecules of the ECM, their function or their arrangement within the ECM. A general

description of the most important ECM molecules and their structure and function will be given below. For a more comprehensive description, the following references are recommended[4,5].

The articular cartilage is organised into four different zones based on structural and functional differences: 1) the superficial zone (SZ), 2) the middle zone (MZ), 3) the deep zone (DZ) and 4) the calcified zone (CZ) (Figure 1).

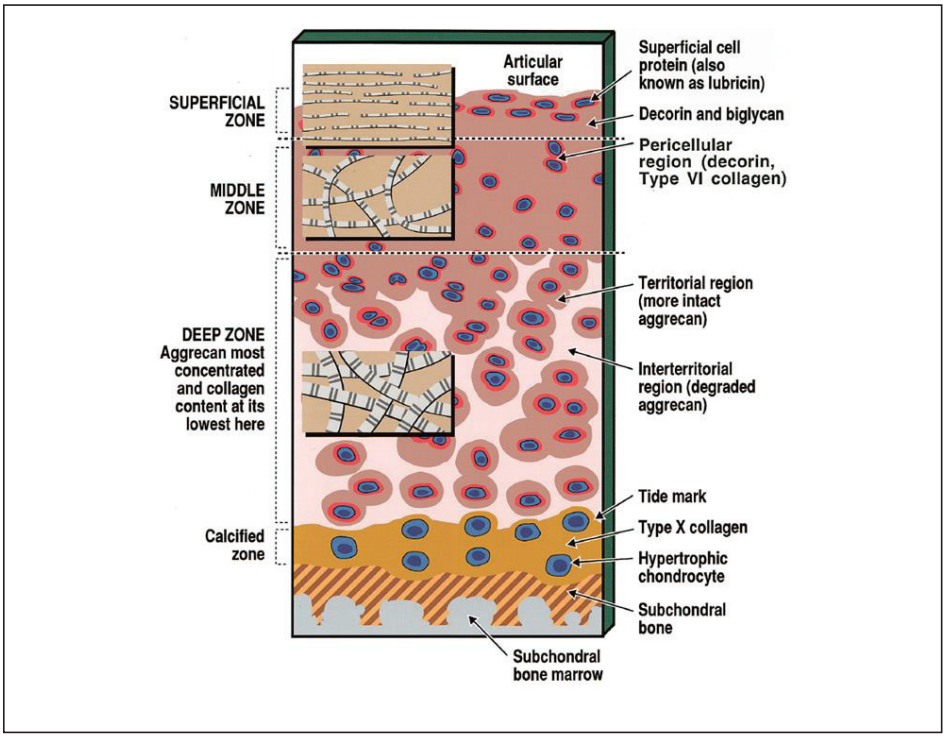

Figure 1. Structure of adult human articular cartilage showing the zonal and regional organisation of the ECM and the relative thickness of the collagen fibrils (from Poole et al., 2001)[5].

The SZ is the surface that lines the synovial joint and is in direct contact with the synovial fluid. At the SZ, the cells are flattened and have a fibroblastoid shape and produce thin collagen fibrils that are oriented horizontally to the articular surface [6]. It has been suggested that the SZ also contain progenitor cells/stem cells that are responsible for growth

during development [7]. In general, there are small amounts of proteoglycans in the SZ, but the chondrocytes produce proteoglycan 4 (also called lubricin) that reduces friction and thereby functions as a lubricant at the articular surface [5,8]. The MZ contains more proteoglycans and has a lower cell density than the SZ. The cells are more rounded, and the collagen fibrils are thicker and more randomly distributed. The DZ has the lowest cell and collagen density, but it has the highest concentration of proteoglycans. The collagen fibrils are thicker and oriented more perpendicularly to the surface, and the chondrocytes are arranged into column-like structures parallel to the collagen fibrils. Below the DZ is the tide mark, a thin interface that separates the non-calcified cartilage from the CZ, which is integrated within the underlying subchondral bone. The chondrocytes in the CZ produce the hypertrophic marker type X collagen, and they are responsible for the calcification of the ECM. In addition to the zonal organisation, there is regional organisation of the ECM (Figure 1). The ECM immediately surrounding the chondrocytes is called the pericellular region. A single chondrocyte and its pericellular region are called a chondron, which is considered to be the smallest metabolic and functional unit of articular cartilage [9]. The ECM outside the pericellular region is called the territorial region, while the ECM most distant to the cells is referred to as the inter-territorial region [4,5,10].

In articular cartilage, the collagen exists as fibrils that are assembled into large collagen fibres. The collagen fibrils consist mainly of type II collagen and small amounts of type XI collagen, while type IX collagen decorates the surface of the type II/XI fibrils and is thought to mediate interaction with other collagen fibrils and with other ECM molecules [11]. The collagen gives the ECM the tensile strength to withstand mechanical stretch, but it also serves as a scaffold where other ECM molecules can be incorporated. The proteoglycan aggrecan and the GAG hyaluronic acid (HA, also known as hyaluronan) are some of the other important molecules in articular cartilage. Aggrecan aggregates are shown in Figure 2.

Several aggrecan proteins are bound perpendicularly to a single HA molecule (blue) in association with a cartilage link protein (dark green) that stabilises the binding. The core protein of aggrecan (light green) itself contains many negatively charged GAGs (red), mostly chondroitin sulfate and smaller amounts of keratin sulfate [12]. Due to the high density of negative charge associated with the GAGs, osmotically active cations, most importantly Na^+, are drawn into the tissue and cause water influx. This creates an osmotic pressure that withstands compressive forces. Together with the tensile strength created by the collagens, the osmotic pressure makes articular cartilage an ideal tissue for load-bearing.

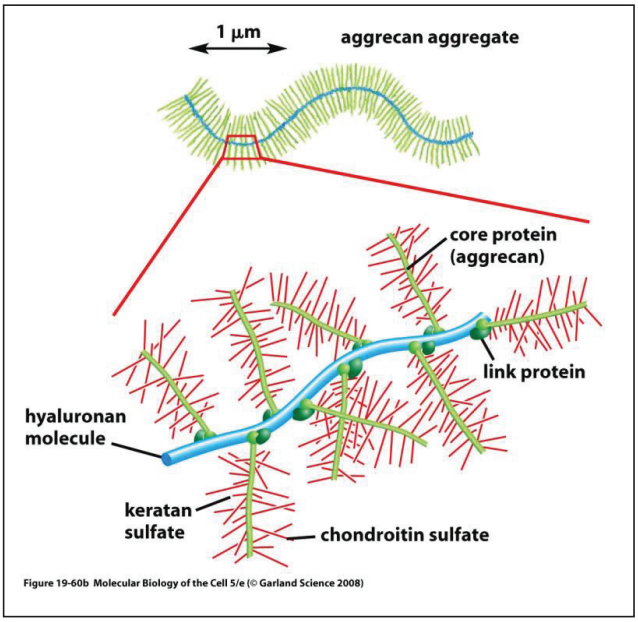

Figure 2. Aggrecan aggregate. See text for details (from Alberts et al., 2008)[12].

The ECM molecules also provide cell-matrix interactions by providing attachment points for cell surface receptors, such as integrins, CD44 and syndecans [13,14]. The binding of the cell surface receptors to ECM ligands activates intracellular signalling pathways that can regulate different biological processes, including cell differentiation, proliferation, shape, orientation,

movement and survival [12,14]. The ECM also binds growth factors and cytokines and can serve as a reservoir that regulates the activity of these important signalling molecules [15].

Chondrogenesis and endochondral bone formation

During development, the articular cartilage is formed through a complex, tightly regulated process called chondrogenesis. Most of what is known about chondrogenesis comes from in vivo studies in chickens and mice. During the development of the long bones, cartilage is formed first and serves as a template for the future bone. Later, through the process of endochondral bone formation (or endochondral ossification), the cartilage is replaced by bone, except at the end of the bones, where it becomes the articular cartilage. The entire process can be divided into five stages (condensation, differentiation, terminal differentiation, calcification/hypertrophy and ossification)[16], schematically shown in Figure 3. In vertebrate limb development, mesenchymal cells first produce an ECM that is rich in HA and type I and II collagen. Then the cells proliferate and migrate to the centre of the limb, leading to aggregation of the cells (condensation). During condensation, the cells stop producing type I collagen, induce hyaluronidase activity to degrade the HA and express cell adhesion molecules such as N-CAM and N-Cadherin. This allows for movement, tight aggregation and cell-cell interactions, which possibly trigger chondrogenesis [17] 2004)[18].

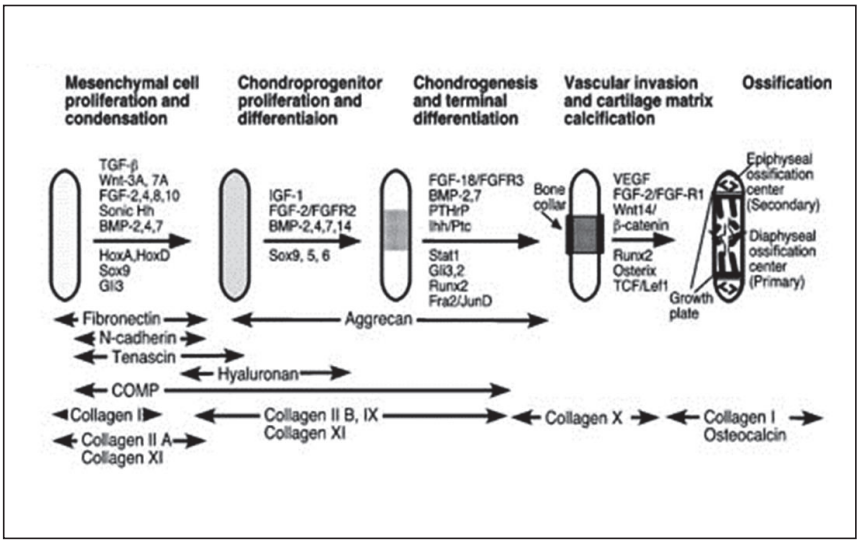

Figure 3. A schematic representation of chondrogenesis and endochondral bone formation. The figure is summarised in the text (from Goldring et al., 2006)[16].

Transforming growth factor beta (TGF-β) is one of the earliest signalling molecules expressed during chondrogenesis and is thought to regulate condensation by inducing expression of fibronectin, which in turn regulates N-CAM and N-cadherin expression [19,20]. TGF-β also induces expression of the transcription factor SRY (sex determining region Y)-box 9 (SOX9), which is essential for condensation, differentiation and cartilage formation [21-24]. At the end of condensation, syndecans bind to fibronectin and downregulate N-CAM expression, which marks the transition from condensation to differentiation [25]. During differentiation, SOX9, together with L-SOX5 and SOX6, stimulate expression of important ECM molecules such as aggrecan, cartilage link protein and type II, IX and XI collagen in a bone morphogenetic protein (BMP) signalling-dependent manner [26]. The importance of L-SOX5 and SOX6 for chondrocyte differentiation was shown by generating double mutant mice lacking both L-SOX5 and SOX6. These mice had severely underdeveloped cartilage

because the chondrocytes were arrested in the condensation stage [27,28]. During terminal differentiation, the chondrocytes further differentiate and mature as they increase the production of types II, IX and XI collagen while the production of fibronectin decreases [29]. As shown in Figure 3, several factors are involved in this step. The balance between BMPs and FGFs (fibroblast growth factors) seems to regulate proliferation and thereby the rate of differentiation/maturation, while parathyroid hormone-like peptide (PTHrP) and Indian hedgehog (IHH) signalling regulate the commitment to hypertrophic differentiation [30]. At the end of terminal differentiation, the mature chondrocytes are thought to be arrested in the cell-cycle before differentiating into hypertrophic chondrocytes. During hypertrophy, the cells increase in size and gene expression shifts from the production of type II collagen to the production of type X collagen (hypertrophic marker). Other molecules that are associated with hypertrophy are vascular endothelial growth factor (VEGF), which attracts blood vessels, matrix metallopeptidase (MMP) 13 and MMP9, which remodel the ECM, possibly by degrading type II collagen and aggrecan, and alkaline phosphatase (ALPL), which is involved in calcification of the ECM [31-33]. The transcription factor runt-related transcription factor 2 (RUNX2) has an important role in this process as it regulates VEGF, MMP13 and ALPL, possibly explaining why endochondral ossification is blocked in RUNX2 knockout mice [34-37]. At a late stage of hypertrophy, osteoclasts and osteoblasts are recruited, and the mineralised cartilage ECM is removed and replaced by bone while the hypertrophic ACs undergo apoptosis [16]. However, the cartilage at the end of the bones does not undergo hypertrophy/ossification and is not replaced by bone; this is the articular cartilage and it persists throughout life. The articular cartilage can therefore be referred to as permanent cartilage, while the cartilage undergoing ossification can be referred to as transient cartilage [38]. The factors regulating the fate of permanent and transient cartilage are not fully known. However, the balance of SOX9 and RUNX2 may determine which part of the cartilage

become bone and which remains cartilage. SOX9 is not expressed in hypertrophic chondrocytes, while RUNX2 is essential for hypertrophy [37,39]. Additionally, in vivo studies have shown that RUNX2 regulates the expression of two different splice variants of the transcription factor ETS related gene (ERG) whose expression is important for regulating the development of chondrocytes into permanent or transient cartilage [38,40-42].

Cartilage injuries and treatment

Cartilage injuries

Because of the highly specialised nature of articular cartilage, the integrity of the ECM is crucial for the health and function of the tissue. Once injured, the articular cartilage has a poor healing capacity due to the lack of vascularisation and the fact that it is almost impossible for the cells to migrate to injured areas because of the high density of the ECM. This feature makes damage and diseases of the articular cartilage one of the leading causes of chronic disability in developed countries [43]. Most articular cartilage injuries are a result of direct mechanical trauma to the cartilage or progressive degeneration as seen in osteoarthritis (OA) [44].

It has been documented that 60-65% of patients undergoing knee arthroscopy have cartilage lesions [45-48] and that sports activity is the most commonly associated event [48]. Focal cartilage lesions not only give rise to pain, but such lesions will enlarge with time and in many cases contribute to the development of OA [44]. OA is a multifactorial disease of the whole joint that leads to degradation of the articular cartilage. The exact causes of OA are not known, but there are many factors, such as age, blunt trauma, obesity, inflammation, diabetes, genetics and joint pathologies, associated with the development of OA [44]. When the articular cartilage in the joint is degraded, the subchondral bone will be exposed and the bone surfaces

will rub against each other. This results in inflammation, pain, swelling and stiffness of the joint. In addition to impairing the quality of life for millions of people, OA is also an economic burden for society. In 2003, the total cost of arthritis and other rheumatic conditions in the United States was estimated to be $128 billion [49].

Treatment

The ultimate goal of cartilage repair is to produce a tissue that has identical properties to native cartilage and that integrates with the surrounding tissue of the lesion. Many surgical techniques have been developed, but so far none of the techniques fulfils these requirements [44,50]. The repair tissue often consists of fibrocartilage, bone, hyaline-like tissue or a mixture of these tissues [51,52]. Although such treatments do not result in a perfect repair tissue, many patients experience improved functionality and relief of pain. This is the main reason for performing these treatments today. Autologous chondrocyte implantation (ACI), or autologous chondrocyte transplantation, is the most widely used cell-based procedure for cartilage repair. Because ACI is the relevant treatment procedure for this thesis, a description will be given below. For a detailed discussion of the other therapeutic strategies, the following review and book are recommended [44,50].

Autologous chondrocyte implantation

In 1994, Brittberg et al., published the first paper on cartilage treatment using ACI [53]. Since then, more than 35,000 patients have been treated using this procedure [44]. Figure 4 shows the classical ACI technique.

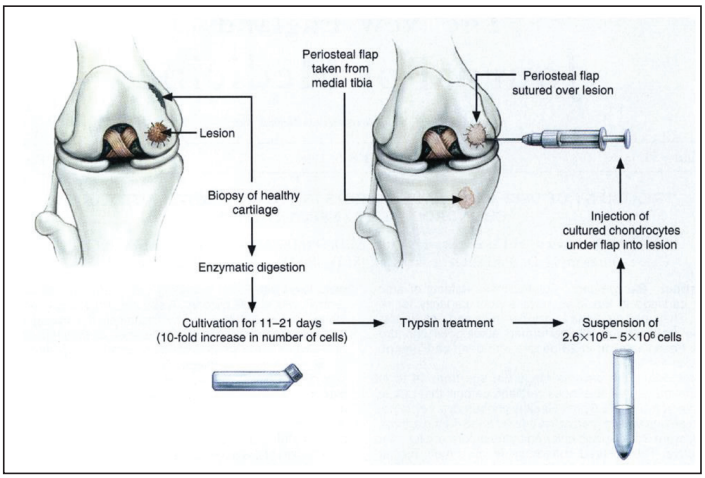

Figure 4. Autologous chondrocyte implantation procedure. The figure is explained in the text (from Brittberg et al., 1994)[53].

A small cartilage biopsy from a non-weight bearing area of the knee (such as the superior and the lateral intercondylar notch or from the edges of the lateral or medial femoral condyles) is harvested and enzyme-treated to release the cells from the ECM. The cells are then expanded (cultured) to obtain a sufficient number to be used for implantation. The cells are then injected into the lesion under a periosteal flap [53]. This process represents the first generation of ACI. However, the periosteal flap may lead to hypertrophy and periosteal delamination. To overcome these problems, researchers have developed a second generation of ACI where the periosteal flap has been replaced by a collagenous membrane (collagen-covered ACI - CACI). CACI results in less hypertrophy compared with the first-generation ACI. Otherwise, the clinical improvement is the same [54]. To further improve the ACI procedure, a third generation of ACI has been developed. This procedure involves seeding cultured ACs onto a collagen membrane (matrix-induced ACI – MACI) or the use of cultured ACs within other three-dimensional scaffolds (such as Hyalograft C, Novocart 3D, Cartipatch and BioSeed-C) [54]. The third-generation ACI has been shown to yield good or excellent results in clinical

trials, but there is no difference between CACI and MACI when comparing the clinical, arthroscopic and histological outcomes [55].

As shown in Figure 4, ACI requires ex vivo monolayer expansion of the patient's own ACs. Monolayer expansion of the cells leads to dedifferentiation. This means that the cells lose their phenotype, and synthesis of the hyaline cartilage-specific proteins, such as type II collagen and aggrecan, is replaced by synthesis of type I collagen and versican, respectively [56,57]. Type I collagen and versican are components of fibrocartilage. Therefore, transplantation of dedifferentiated ACs results in a repair tissue consisting of fibrocartilage or a mix of fibrocartilage and hyaline cartilage [52]. Although this repair tissue improves functionality and alleviates symptoms, it does not have the mechanical and osmotic properties of hyaline cartilage and it will eventually degrade [44]. This is a major limitation for the use of ACs in ACI. The reason for dedifferentiation is not fully known, but it is thought to involve the actin cytoskeleton and the spreading or flattening of the cells when they attach to the culture surface during monolayer expansion [58,59]. Understanding the mechanism behind dedifferentiation may lead to new strategies for ex vivo expansion in which the ACs do not dedifferentiate, thus improving the quality of the repair tissue and the clinical outcome of ACI. Other factors, such as age, ligament stability, meniscus damage and the size of the defect also affect the quality of the repair tissue [44]. Another limitation of ACI is the need for two surgical interventions, one to harvest the biopsy and one to transplant the cultured cells (Figure 4). Harvesting the biopsy also leads to donor site morbidity, but it does not seem to create problems during ACI in the knee. However, most studies using MRI do not mention donor-site morbidity and there are no published data on the anatomy or histology of the donor-site [60].

Introducing another cell source may not only improve the clinical outcome of ACI, but it will also avoid the need to harvest a biopsy from the knee and prevent potential problems

related to donor-site morbidity. Stem cells are an alternative cell source for cartilage repair. In the next section, I will describe some of the basic biology of stem cells and then focus on mesenchymal stem cells (MSCs) as candidates for cartilage repair.

Stem cells

Stem cell research holds great promise for regenerative medicine and tissue engineering in medical research, including cartilage repair. A stem cell, by definition, is an undifferentiated (or unspecialised) cell that can produce both new stem cells (self-renewal) and cells that commit to differentiation (specialisation) (Figure 5). The daughter cells committed to differentiation first become precursor cells. These can divide symmetrically to form more precursor cells, or differentiate further to become tissue specific, end-differentiated cells [61].

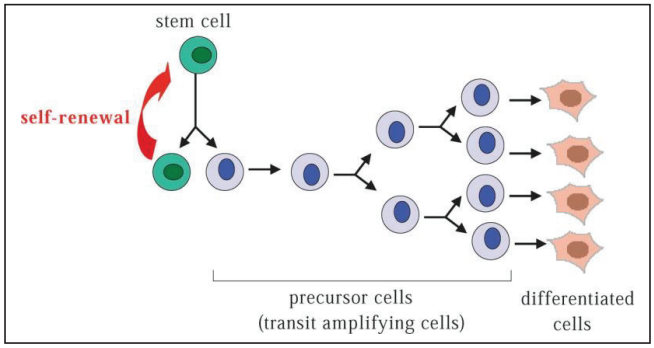

Figure 5. Definition of a stem cell. See text for explanation (from Raff, 2003)[61].

Stem cell differentiation is the process where an undifferentiated stem cell changes its features to become a more specialised cell, such as a neuron, chondrocyte or a muscle cell. In vivo, stem cells usually remain undifferentiated and in a slowly proliferating state until they receive a signal that causes them to commit to differentiate [62]. The differentiation signal triggers the

altered expression of genes involved in the cell cycle and tissue development. Depending on the signal, some cells will divide and produce identical daughter cells to maintain the stem cell pool, while others will divide to generate differentiated cells.

Classification of stem cells

Some stem cells can produce a variety of differentiated cells [63], while other stem cells can produce only a few or one type of differentiated cells [64]. The differentiation potential largely depends on where in the body or from which stage in development the stem cells arise. Stem cell researchers typically draw a distinction between embryonic stem cells and adult stem cells.

Embryonic stem cells

After fertilisation, the egg is referred to as a zygote. The zygote is totipotent, meaning that it is capable of giving rise to all the cell types that are necessary to form an individual, including those that do not form part of the embryo, such as the cells of the placenta and umbilical cord. Between four to six days after fertilisation, the zygote has developed into a blastocyst [65]. The blastocyst contains two layers of cells, an outer layer called the trophectoderm and an inner layer called the inner cell mass (ICM). The cells of the trophectoderm are involved in implantation and the formation of the placenta, while the ICM develops into the three germs layers (ectoderm, endoderm, and mesoderm) that eventually give rise to all the cells and organs in the adult body. Embryonic stem cells (ESCs) are established by removing the ICM from the blastocyst and growing the cells in vitro (Figure 6) [63].

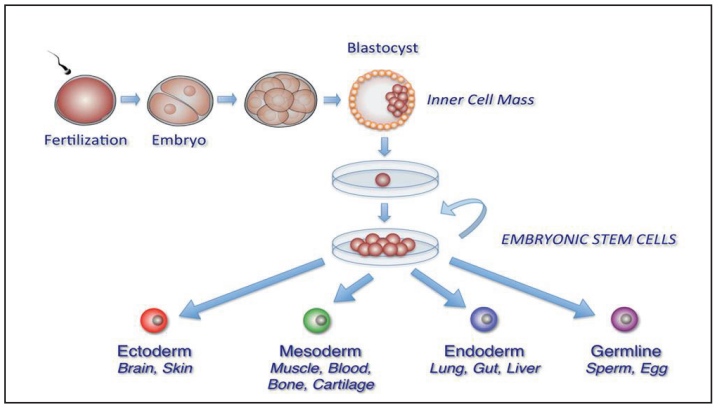

Figure 6. Derivation of embryonic stem cells (modified from Yabut et al., 2011)[66].

ESCs are pluripotent, meaning that they are undifferentiated cells that have the potential to give rise to all the different cells in the adult body. Pluripotency can be demonstrated by injecting the cells under the skin of immune-deficient mice where they will form teratomas (noncancerous tumours) consisting of cells of all three germ layers [67]. Alternatively, the cells can be established in three-dimensional aggregates called embryoid bodies that also give rise to cells of the three germ layers [68]. Studies of ESCs show that relatively few genes are required for self-renewal. The expression of OCT4, SOX2, KLF4 and cMYC all decrease during ESC differentiation [69-73], and in 2006, Yamanaka and colleagues showed that forced expression of these four genes was sufficient to re-programme differentiated cells and turn them into self-renewing ESC-like cells [74]. These cells are called induced pluripotent stem cells (iPSCs), and with John Gurdon Yamanaka won the Nobel Prize in Physiology and Medicine 2012 for this excellent work.

Adult stem cells

Stem cells are present in most, if not all, organs in the body, and they are referred to as adult stem cells (ASCs). An important role of ASCs is to maintain tissue homeostasis and repair by replacing apoptotic cells as a part of normal tissue/cell turnover and by replacing damaged cells following injury. In the tissues, the ASCs have their own specialised environment called the stem cell niche [62,75]. The adult stem cell niche is poorly defined, but it consists of all the components of the microenvironment in which the ASCs reside, such as other cells, ECM molecules, oxygen tension and factors secreted by cells. Both extrinsic signals from the niche and intrinsic signals arising within the stem cells control the balance between self-renewal and differentiation. In this way, the ASCs maintain a pool of stem cells (self-renewal) and serve as a reservoir to replace cells (differentiation) when needed. ASCs have a more restricted differentiation potential than ESCs. Typically, ASCs differentiate into cell types that correspond to the organ or tissue from which they originated. This restricted differentiation potential is often referred to as multipotency. It has been reported that ASCs can also differentiate into lineages that are different from their organ/tissue of origin [76,77].

Mesenchymal stem cells

MSCs, also called multipotent mesenchymal stromal cells, are multipotent cells that can be isolated from many tissues including adipose tissue [78,79], bone marrow [80], teeth [81], periosteum [82], synovial membrane [83], placenta [84], umbilical cord blood and skeletal muscle [85]. MSCs were first described by Friedenstein and colleagues more than four decades ago [80]. Friedenstein isolated cells from bone marrow that adhered to plastic surfaces and formed fibroblast-like colonies (colony forming unit-fibroblasts – CFU-U) when plated at a low density. In vivo transplantation demonstrated that a single cell could give rise to bone,

cartilage, adipose and fibrous tissue [80,86,87]. MSCs consist of a heterogeneous group of cells with varying proliferation and differentiation potentials [88,89]. Studies have shown that these cells can be cultured for many passages in vitro and that they can differentiate into several mesodermal cell types, such as osteocytes, adipocytes, chondrocytes and myoblasts (Figure 7) [90-92].

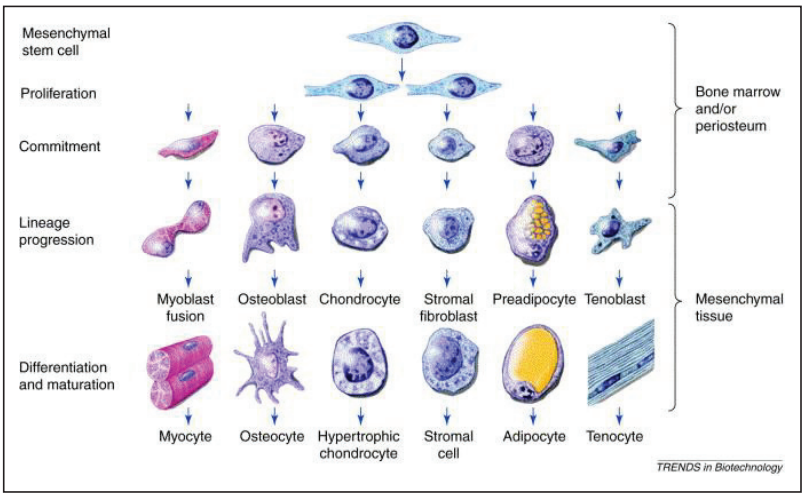

Figure 7. Differentiation potential of MSCs (from Risbud et al., 2002)[92].

Characterisation of MSCs

Because laboratories around the world have used different protocols for the isolation and expansion, as well as different approaches for characterisation of these cells, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has proposed three criteria to define human MSCs [93]. First, the cells must adhere to plastic surfaces. Second, MSCs should express certain cell surface markers, and 95% or more of the cell population should express CD105 (endoglin), CD73 (ectonucleotidase) and CD90 (thy-1). Because MSCs are isolated from tissues containing many different cells, the MSC cultures may be contaminated by other cell types, such as

hematopoietic cells. Therefore, it is recommended that cultured MSCs do not express (less than 2%) the hematopoietic stem cell marker CD34, the hematopoietic marker CD45, one of the two macrophage markers CD14 or CD11b and one of the two B-cell markers CD79α or CD19. MSCs should not express HLA-DR, but this molecule can be induced by stimulating the cells with interferon-γ [(94)]. The third criterion requires that the cells must be able to differentiate into osteocytes, adipocytes and chondrocytes in vitro.

In vivo localisation of MSCs

MSCs can be isolated from many tissues, but the exact origin and in vivo niche of MSCs are not fully known. However, MSCs in the bone marrow surround small blood vessels (sinusoids) and have a very important role in the niche of haematopoietic stem cells (HSCs) [(95)]. MSCs express HSC regulatory molecules, such as CXCL12, which facilitates homing of hematopoietic progenitors and hematopoiesis [(96)]. Transplantation of MSCs has also shown that these cells can organise the hematopoietic microenvironment [(97)]). Recently, MSCs have been shown to have a perivascular location in several tissues, suggesting that MSCs and pericytes (cells that wrap around endothelial cells in capillaries) are the same cells [(98-100)]. If MSCs are pericytes, it would explain why MSCs are found in many tissues and it would also allow MSCs to easily access most tissues/organs for repair/tissue homeostasis. Another possibility is that stem cells from various tissues acquire MSC characteristics when established in vitro [(100)]. In vivo studies have shown that MSCs can migrate to various organs in the body after transplantation [(101,102)], and Rochefort et al. demonstrated mobilisation of MSCs into the peripheral blood when rats were exposed to hypoxia [(103)]. In contrast to the MSC/pericyte theory, this finding suggests that MSCs are located in one or a few organs and that they can enter the circulation and migrate to other tissues after stimulation. However, the difficulty of isolating MSCs from the peripheral blood argues against this theory.

MSC self-renewal

Understanding signalling pathways and the factors controlling self-renewal and differentiation of MSCs is important for the clinical use of these cells. Clonal studies of MSCs derived from human umbilical cord demonstrated a hierarchical schema for self-renewal and differentiation [(104)]. The authors observed no difference in CFU-U formation between parent cells and daughter cells (clones), suggesting self-renewal and maintenance of the stem cell pool. Further support for self-renewal was based on the fact that daughter clones maintained the ability to differentiate after 40 cell doublings. There has been a debate regarding whether MSCs are able to self-renew, and there is a lack of robust and reproducible methods for assaying self-renewal in these cells. However, in vivo studies have shown that colony forming CD146+ MSCs are capable of organising a hematopoietic environment (heterotopic bone marrow) in mice and that the same CD146+ cell population can be isolated from the heterotopic bone marrow and subsequently passaged and assayed for colony formation [(97)]. Another group demonstrated that Nestin+ MSCs were able to self-renew and give rise to heterotopic bone marrow in serial transplantations [(105)]. Although these studies provide some evidence for the self-renewal of MSCs, there are no standardised or robust assays to confirm the self-renewal of MSCs. The signalling pathways regulating self-renewal in MSCs are not well understood. As already mentioned, OCT4, SOX2, KLF4 and CMYC are important for self-renewal of ESCs. Human bone marrow MSCs do not express *SOX2*, but they do express low levels of *OCT4* and *NANOG* (another important "pluripotent gene") and moderate/high levels of *CMYC* and *KLF4* (Master thesis: Tommy Aleksander Karlsen, 2007, University of Oslo, Norway). The roles of these genes in MSCs are unclear, but knockdown of *OCT4* and *NANOG* inhibited proliferation and differentiation of MSCs, suggesting that these genes have a role in self-renewal [(106)]. Leukemia inhibitory factor (LIF) maintains the undifferentiated state of MSCs [(107)]). The mechanism is not known, but in mouse ESCs LIF controls self-

renewal through a CMYC-dependent mechanism [73]. Fibroblast growth factor (FGF) 2 increases the lifespan and differentiation capability of MSCs from a range of species when cultured as a monolayer, suggesting that FGFs have an important role in the self-renewal of MSCs [108]. Other cytokines and signalling pathways, including WNT (Wingless) [109], the WNT inhibitor Dickkopf-1 [110], tumour necrosis factor-α, platelet-derived growth factor receptor β [111], polycomb chromatin-associated proteins [112], and Notch [113] all have roles in the determination of cell fate in MSCs, but the mechanisms are not fully understood.

Chondrogenic differentiation

As shown in Figure 7, MSCs can differentiate into several lineages, including chondrocytes. For several years, researchers have used different growth factors and three-dimensional cultures to induce chondrogenesis of MSCs in vitro. Some of the main strategies of chondrogenic differentiation will be described here.

In vitro chondrogenesis using pellet cultures

The pellet culture is a simple procedure where the cells are centrifuged and cultured as a pellet in the bottom of a tube. This is analogous to the condensation phase in vivo, where cell-cell contact is established. The pellet culture has been used to study many aspects of chondrogenesis, including condensation, hypertrophy and the influence of oxygen concentration and mechanical hydrostatic pressure [114-118]. The pellet culture was first described in 1960 in an attempt to maintain the differentiated state of chicken embryonic ACs in culture [56]. Seven years later, the pellet culture was applied to human ACs [119]. Since the studies by Friedenstein in the 1970s, it was clear that MSCs were capable of differentiating into chondrocytes in vivo, but it was not until 1998 that in vitro chondrogenesis of MSCs was

demonstrated [120]. In contrast to ACs, the MSCs did not form aggregates unless a defined culture medium containing TGF-β and/or dexamethasone was used. Sekiya et al. improved chondrogenesis by adding BMPs to the chondrogenic induction medium [121,122]. Today, several combinations of growth factors and hormones are used in the chondrogenic induction medium. The most commonly used are TGF-β, BMPs and dexamethasone [121-123], but in some protocols, FGF2, IGF-I and/or PTHrP are also used [124,125]. TGF-β and BMPs belong to the TGF-β superfamily and have a broad range of biological activities, including proliferation, differentiation and apoptosis [4]. In mammals, there are three different TGF-β proteins (TGF-β1-3), and they elicit their biological functions by binding to TGF-β-receptors, resulting in phosphorylation of Smad proteins. The phosphorylated Smad proteins then translocate to the nucleus where they regulate gene expression [12]. The BMPs are a large group of proteins (at least 20) that act through the same receptors as TGF-β but engage another set of Smad proteins [12]. For a more comprehensive description of TGF-β, BMPs and other growth factors, as well as their role in chondrogenesis/cartilage, the following book is recommended [4].

Micromass culture is another method that is very similar to the pellet culture method. Instead of making pellets by centrifugation, the cells are suspended at a very high cell density in medium and allowed to self-assemble into small aggregates [126,127].

In vitro chondrogenesis using three-dimensional scaffolds

Pellet cultures often contain necrotic cells at the centre of the pellet, possibly as a result of inadequate diffusion of nutrients [128]. The pellets also have a very small size (~200,000 cells/pellet), making it difficult to obtain a large number of differentiated cells. Thus, the clinical use of pellet cultures has limitations, and this method is therefore not used for these purposes [129]. As described in the section "Autologous chondrocyte implantation" (page 19), cultured chondrocytes can be seeded or embedded in three-dimensional scaffolds before

implantation. This principle has been applied for in vitro chondrogenesis of both MSCs and ACs.

There are various types of three-dimensional scaffolds, and new scaffolds are constantly being developed. Scaffolds can broadly be divided into three groups [50,130]. The first group consists of protein-based polymers such as collagen, fibrin and gelatin. The second group corresponds to carbohydrate-based polymers, such as alginate, hyaluronic acid, agarose and chitosan. Artificial, or synthetic, polymers constitute the third group and include scaffolds made of materials such as carbon fibres, Dacron, polyester-urethane and hydroxyapatite. It is also possible to make scaffolds consisting of several types of materials or to incorporate molecules for attachment and/or cell signalling purposes. In general, scaffolds should support cell viability and proliferation, be biocompatible, biodegradable, provide sufficient structural and mechanical support, provide a uniform distribution of cells and contain large enough pores to allow the diffusion of nutrients and the removal of waste products. For tissue engineering purposes, the scaffold material should also allow for integration of the newly synthesised tissue with the adjacent tissue [130]. A large number of scaffolds have been used to support chondrogenesis. Still, there are no scaffolds (or other methods) available today that promote the formation of perfect articular cartilage in vitro or in vivo, reflecting the complex nature of this tissue [131]. The goal of combining cells and scaffolds is to provide an environment that mimics the effect of the native environment surrounding the cells in vivo. Reconstructing the in vivo environment of ACs within a scaffold is a difficult, if not impossible, task. However, studies have shown that both MSCs and dedifferentiated ACs are capable of surviving and differentiating toward the chondrogenic lineage in a wide range of scaffolds [130]. Hopefully, mimicking some aspects of the native environment will be sufficient to support the formation of articular cartilage with properties comparable to the native tissue. This will eventually improve the clinical outcome for patients in the future.

Immunomodulatory effects of MSCs

MSCs are regarded as immune privileged cells based on observations that allogeneic MSCs avoid immune recognition [132]. MSCs have also been used for treatment of graft-versus-host disease in humans with promising results [133,134]. In vitro studies show that MSCs have the capability to suppress the function of T-cells, B-cells, natural killer cells, inhibit maturation of dendritic cells and regulate activation of macrophages [135,136]. MSCs exert their immunosuppressive function by secreting soluble factors, such as indoleamine 2,3-dioxygenase, nitric oxide and prostaglandin E2 and through direct cell-cell contact [135,136]. Ren et al. demonstrated for example that an increased expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) correlated with the immunosuppressive capacity of MSCs, while blocking or deleting these adhesion molecules inhibited immunosuppression [137].

microRNAs

MicroRNAs (miRNAs) are small double-stranded RNA molecules that regulate gene expression by targeting complementary nucleic acid sequences. miRNAs are transcribed as large transcripts called primary miRNA (pri-miRNA) (Figure 8). In the nucleus, pri-miRNAs are recognised and processed by the Drosha microprocessor complex [138]. The resulting product is called precursor miRNA (pre-miRNA) and contains a two-nucleotide overhang at its 3'-end. The Exportin complex then transports the pre-miRNAs into the cytoplasm where Dicer cleaves the pre-miRNA into mature double-stranded miRNA molecules approximately 21-23 nucleotides in length [139]. One or both of the strands is incorporated into the RISC complex where it will interact with complementary mRNA molecules by base pairing, resulting in either degradation of the mRNA or translational repression [140,141]. The two

strands of the pre-miRNA may give rise to two mature miRNAs. These are given the suffix "5p" (from the 5' arm) or "3p" (from the 3'arm). The suffix "5p" or "3p" is not always used in the literature. In this thesis, the lack of "5p" and "3p" refers to the mature miRNA from the 5'arm (5p). For further information on the nomenclature of miRNAs, please visit the miRbase database (http://www.mirbase.org/).

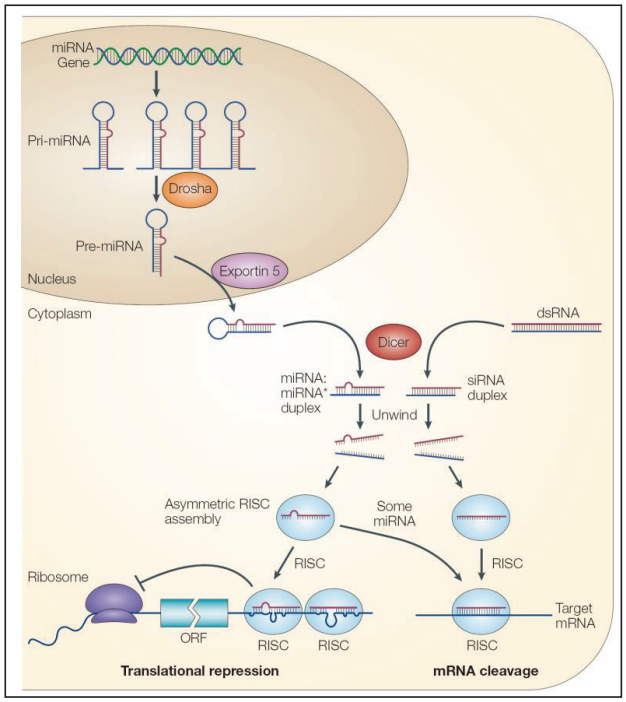

Figure 8. miRNA biogenesis. The figure is explained in the text (from He et al., 2004)[142].

Recently, miRNAs have also been shown to enhance gene expression. In some situations, this may occur through activation rather than repression of translation [143,144]. In other situations, miRNAs may bind to promoters with complementary sequences to induce or repress transcription [145,146]. One miRNA may have more than one hundred different mRNA molecules as targets [147], and experiments have demonstrated that a single miRNA can affect the expression of hundreds of proteins, directly or indirectly [148]. Today, more than 2000

miRNAs have been discovered in humans (www.miRbase.org), and estimations suggest that 60% of our genes are regulated by microRNAs [149]. Thus, miRNAs are likely to be involved in most biological processes.

Role of miRNAs in cartilage, chondrogenesis and osteoarthritis

MiRNAs are necessary for the development of normal cartilage, as mice with chondrocytes lacking the Dicer gene exhibit severe skeletal defects [150]. Studies in mouse and zebrafish embryos have identified miR-140 as a cartilage-specific miRNA [151,152]. MiR-140 is located in an intron in the *WWP2* gene and is induced by SOX9 [153,154]. Recently, it was demonstrated that SOX5/6 increases the transcription of miR-140 by boosting dimerisation and DNA binding of SOX9 [155]. The importance of miR-140 in cartilage development has been shown in in vivo studies. Miyaki et al. showed that miR-140-deficient mice have an OA-like pathology and a shorter skeleton compared to wild-type mice, while transgenic mice overexpressing miR-140 were resistant to antigen-induced arthritis [156]. It was also demonstrated that miR-140 provided resistance to proteoglycan and type II collagen loss and that ADAMTS5 (a matrix-degrading protease) was a direct target of miR-140, partially explaining the protective role of miR-140 against OA progression. In another in vivo study, Nakamura et al. showed that the loss of miR-140 impaired endochondral bone formation by accelerating hypertrophic differentiation. Further, it was demonstrated that miR-140 could modulate BMP signalling by targeting *DNPEP* [157]. In micromass cultures miR-140 regulates proliferation by targeting the transcription factor SP1 [153]. In vivo miR-140 is expressed in proliferating chondrocytes supporting the regulatory effect of miR-140 on proliferation [156]. *PDGFRA* is another target of miR-140 and is important in palatogenesis in zebrafish [158], while the TGF-β superfamily modulator *SMAD3* has been shown to be a target in C3H10T1/2 cells [159]. Other validated targets of miR-140 are *BMP2, CXCL12* and *HDAC4* [151,160,161].

In addition to miR-140, other miRNA may also have important roles during cartilage development. *SOX9* has been verified as a target of miR-145 in two studies [162,163]. In bovine articular cartilage, miR-222 plays a role in mechanotransduction [164], while miR-221 has been reported to be a negative regulator of chondrogenesis in chick limb mesenchymal cells [165]. Overexpression of miR-675 positively regulates type II collagen expression possibly via a SOX9-dependent mechanism [166]. MiR-199a inhibits early BMP2-induced chondrogenesis in C3H10T1/2 cells by targeting SMAD1 [167]. In rats, several miRNAs were shown to be upregulated during the development of articular cartilage. These include miR-25, miR-26a, miR-140, miR-150, miR-181a and miR-210, while miR-1 was downregulated [168]. In another study miR-1 was found to be repressed during hypertrophic differentiation and to inhibit the expression of aggrecan [169]. MiR-365 is highly expressed in hypertrophic chondrocytes and stimulates type X collagen [170]. Besides miR-140, the dysregulation of several miRNAs is also associated with OA, including the suppression of miR-25, miR-26a, miR-27a, miR-27b, miR-29a, miR-210, miR-337, miR-373 and the upregulation of miR-9, miR-16, miR-22, miR-23b, miR-30b, miR-34a, miR-34b, miR-103, miR-223, miR-377, miR-455, miR-483 and miR-509 [171-173]. Mir-146a may also be involved in OA, but it seems to be expressed differently depending on the developmental stage of OA [174].

Potential clinical applications of miRNAs

Endogenous miRNAs regulate many genes, and it is possible for one miRNA to regulate many genes belonging to the same biological pathway. One mRNA may also be targeted by several miRNAs [175-177]. Thus, it is not unexpected that the dysregulation of miRNAs is associated with several diseases, including OA [156,173]. In animal models, synthetic miRNA mimics and inhibitors have demonstrated promising results. Administration of a let-7b mimic reduced tumour formation in mice, while treatment of hepatitis C virus (HCV) infected

chimpanzees with a miR-122 inhibitor (HCV replication is miR-122-dependent) reduced HCV viremia and improved liver pathology compared to control animals [178,179]. Because of their important role in gene regulation and their promising results in animal studies, manipulation of endogenous miRNAs has attracted the attention of the biomedical research community, and in 2008, Santaris Parma A/S commenced the first human clinical trial for an miRNA-targeted drug (a miR-122 inhibitor) (www.santaris.com). In 2010, the study was advanced to a phase 2a clinical trial, and the results were announced in November 2011. So far, the drug appears to be both effective and safe for treating HCV patients [180].

One advantage of the use of small synthetic miRNA mimics or inhibitors in therapy is that they do not integrate into the genome and therefore offer greater safety than gene therapy utilising DNA plasmids. Compared to large gene constructs, these small RNA molecules (21-23 nucleotides) are easier to introduce into cells and because they function in the cytoplasm, they do not need to enter the nucleus, thus avoiding the use of complex delivery systems. Another major advantage of manipulating miRNAs is that several target genes belonging to the same biological process can be affected by manipulating only a single miRNA. For example, a tumour-suppressor miRNA may target both anti-apoptotic genes and genes involved in angiogenesis and the cell cycle. Such tumour-suppressor miRNAs are often downregulated in several types of cancers [178,181]. By restoring endogenous miRNAs with synthetic mimics, multiple cancer-genes can be targeted at the same time. This principle can also be used for targeting miRNAs involved in chondrogenesis and the development of OA.

To use miRNA mimics or inhibitors in therapy, it is necessary to understand the biological role of the miRNA to be manipulated. Thus, many in vitro and in vivo experiments have to be performed prior to clinical trials. The usefulness of these studies depends on the efficiency of the miRNA mimic/inhibitor for inducing specific changes in target genes and their downstream mediators. Different delivery methods are used for introducing

mimics/inhibitors to cells. One commonly used method is the use of lipid-based transfection reagents (liposomes). These reagents are made to mimic the properties of biological lipids to ensure fusion with cell membranes and delivery of the nucleic acids into the cell either via the endosomal pathway or by direct delivery into the cytoplasm [182,183]. Both in vivo and in vitro experiments have shown that lipid-based transfection reagents activate the innate immune system [184-186]. RNA molecules may also activate the innate immune system. This occurs by recognizing the RNA by RNA receptors located either in the endosomes or in the cytoplasm [187]. Unintended activation of immune responses by liposomes and/or RNAs can potentially mask or change the cellular response to the synthetic miRNA mimic/inhibitor used and can confuse the interpretation of results. Furthermore, these effects could potentially harm patients. Understanding the molecular mechanisms behind these effects and how to avoid them are therefore important if these reagents are to be employed in clinical trials. However, in certain situations, an immune response may actually be beneficial. This scenario is described in the General discussion section of this thesis.

Cellular receptors for foreign RNA

The mechanisms behind liposome-induced immune response are largely unknown. However, toll-like receptor 4 (TLR4) induces the secretion of interleukins after liposome stimulation [188]. The liposome induced immune response has also been shown to depend on STING (an ER transmembrane protein) [184]. Certain lipid receptors in the cell membrane are known to induce upregulation of immune genes and may also be involved [189,190].

Regarding transfection and immune responses (also referred to here as immunological off-target effects), much more is known about pattern recognition receptors (PRRs) and their role in sensing nucleic acids. PRRs can be divided into membrane-bound PRRs and cytoplasmic PRRs. PRRs are used by the innate immune system to recognise structures called

pathogen-associated molecular patterns (PAMPs) that are shared among related microbes, such as single and double-stranded RNA from viruses and lipopolysaccharides found in the outer membrane of Gram-negative bacteria [191]. After detection of PAMPs, PRRs are activated and initiate an immune response as a defence mechanism against invading microbes [191]. There are many PRRs, and only the PRRs that sense RNA will be introduced here.

Toll-like receptors

TLRs are membrane-bound proteins found at the cell surface and in endosomes. All TLRs have an extracellular domain containing leucine-rich repeats that mediates the detection of PAMPs and an intracellular signalling domain called Toll-interleukin (IL-1) receptor (TIR). In humans, ten TLRs (TLR1-10) have been identified in which TLR3, 7, 8 and 9 are located in the membranes of endosomes where they interact with microbial nucleic acids. The other TLRs are situated in the plasma membrane and recognise PAMPs other than nucleic acids, such as flagellin, peptidoglycan and lipopolysaccharide [191]. Once activated by PAMPs, the TLRs recruit adaptor proteins that in turn initiate a signalling cascade resulting in the production of antiviral factors that are important in the first line of defence against viral infections [191].

The TLRs that respond to RNA are TLR3, TLR7 and TLR8, and they are located in the membranes of endosomes. While TLR3 has double-stranded RNA (dsRNA) as a ligand and signals through the adaptor protein TIR-domain-containing adapter-inducing interferon-β (TRIF), TLR7/8 detects single-stranded RNA (ssRNA) and uses myeloid differentiation primary response 88 (MYD88) as an adaptor protein (Figure 9) [187]. Interestingly, TLR7/8 were also shown to be activated by dsRNA (siRNA duplexes) [191,192]. From the adaptor proteins, the signalling cascade may proceed through different pathways depending on the cell type and which TLR receptor is activated. At the end, all of the TLRs engage transcription

factors involved in the NF-κB and IRF pathways, leading to the expression of antiviral factors such as proinflammatory cytokines and type I interferons to inhibit the replication and spreading of viruses (Figure 9) [191].

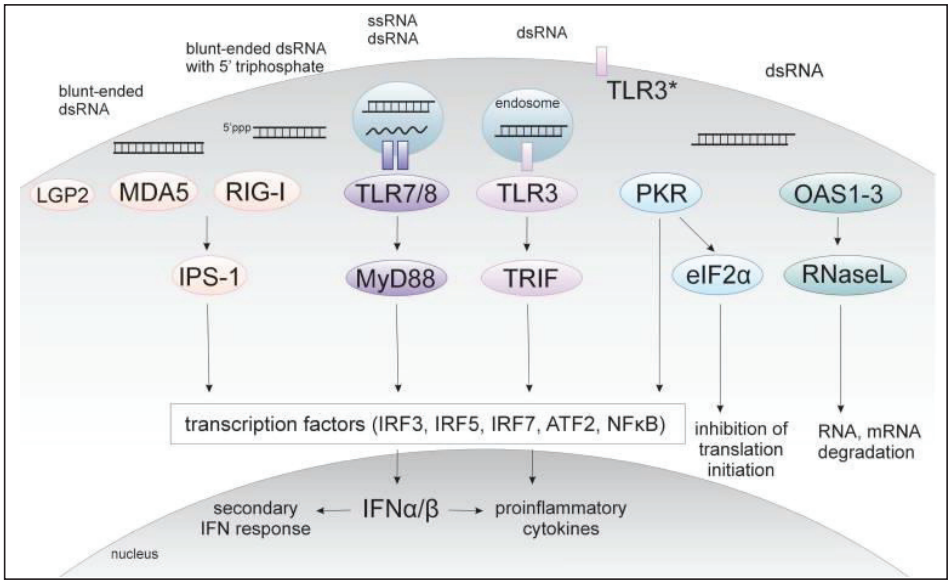

Figure 9. Cellular sensors, adaptor molecules and cytokines involved in innate immune responses to foreign RNA (from Olejniczak et al., 2010)[187].

The retinoic acid-inducible gene-I-like receptors

Unlike TLRs, which are membrane-bound PRRs, the retinoic acid-inducible gene-I-like receptors (RLRs) are cytoplasmic PRRs. The RLRs constitute three members of the DExH/D-box RNA helicase family and include retinoic acid-inducible gene-I (RIG-I), melanoma associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) [193]). RLRs also function as sensors of viral RNA. RIG-I binds preferentially to short dsRNA, while MDA5 binds to long dsRNA (1-10 kb in length) [194,195]. The function of LGP2 in virus recognition is controversial, and it has been reported to act as both a negative and positive regulator of RIG-I [196,197].

RIG-I, the dsRNA receptor investigated in paper III, contains two N-terminal caspase-recruiting domain (CARD)-like domains, a central helicase domain and a C-terminal regulatory domain (RD) (Figure 10) [198]. The CARD domains interact with the downstream mediator mitochondrial antiviral signalling (MAVS, also known as IPS-1), leading to activation of the same transcription factors involved in TLR signalling (NF-κB and the IRF pathways) and the expression of proinflammatory cytokines and type I interferons (see Figure 9) to ensure that the infecting virus is destroyed.

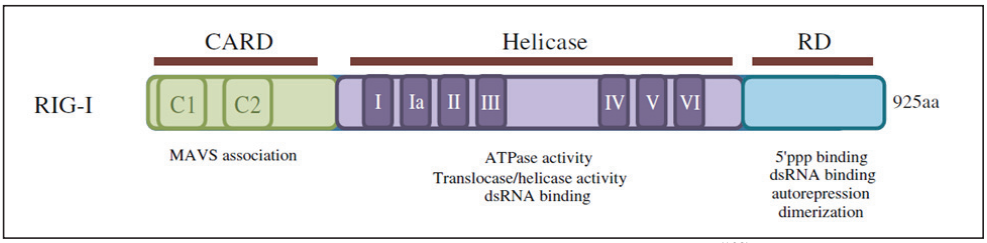

Figure 10. Structure of RIG-I. See text for details (from Baum et al., 2010)[198].

The helicase domain is involved in the recognition and unwinding of the foreign RNA, and it also appears to cooperate with the RD domain to optimise the binding to the RNA [199-201]. In non-infected cells, RIG-I has been suggested to be folded in such a way that the RD domain interacts with the CARD domains and prevents them from signalling through MAVS [202]. During virus infection, a conformational change results in displacement of the repressing RD domain, thereby enabling interaction of CARDS with MAVS and subsequent signalling. RIG-I activity is also negatively and positively regulated by phosphorylation and ubiquitination, respectively [203,204]. In addition, RIG-I is upregulated, though not activated, by cytokines, such as type I interferons, IL-1β and TNF-α [193].

Generally, short dsRNAs rich in GU or polyU motifs have been shown to be more likely to induce immune responses via RIG-I [205]. In addition, RIG-I binds short, blunt-ended uncapped dsRNA, preferably with 5'triphosphate (ppp) groups, though RIG-I has also been

shown to bind dsRNA without 5'ppp and ssRNA containing 5'ppp [200,206-208]. The lack of a cap is typical of viral sequences, and this feature allows RIG-I to discriminate between self and viral dsRNAs [209]. dsRNA containing overhangs, such as endogenous miRNAs, may inhibit immune gene induction through RIG-I, thus introducing another mechanism by which the cell can discriminate between self and non-self dsRNAs [200]. However, blunt ends are not always a requirement for RIG-I activation, as shown in paper III, where both blunt ends and overhangs stimulated an immune response via RIG-I. The results presented in paper III also show that synthetic miR-145 induced an immune response in MSCs via RIG-I when it was delivered by liposomes, but this was not the case when smiR-145 was delivered directly into the cytoplasm by electroporation, suggesting that RIG-I depends on liposome delivery in certain situations (paper III). Thus, the choice of delivery vehicle is crucial for avoiding immunological off-target effects that can complicate the interpretation of results from in vitro and in vivo transfection experiments.

Aims of the study

The overall aim of this thesis was to investigate the role of miRNAs during dedifferentiation and chondrogenic differentiation of ACs and MSCs, respectively.

Thus, the specific aims in this thesis were

- To investigate the global miRNA changes during dedifferentiation of ACs to identify possible miRNAs important for cartilage development and repair.

- To compare the global miRNA expression profile of uncultured ACs with that of chondrogenically differentiated MSCs.

- To investigate the role of miRNAs that showed a reciprocal relationship during dedifferentiation/differentiation and to reveal their possible targets.

Summary of results

Paper I

In this study, we isolated human primary uncultured ACs. When these cells were allowed to proliferate within their own ECM, they began to produce hyaline ECM molecules similar to embryological chondroblasts. Upon continued culture, these cells spread onto the plastic surface and dedifferentiated. Thus, the ACs went through three stages during 28 days of in vitro culture: (1) primary, uncultured non-proliferating ACs (day 0); (2) the chondroblast-like stage (day 7–14); and (3) the dedifferentiation stage at the end of the culture. The three cell populations were investigated with respect to their expression of a large number of genes, miRNAs and proteins related to chondrogenesis. Gene expression was quantified by RT-qPCR, miRNAs were evaluated by miRNA arrays, and protein synthesis was investigated by extra- and intracellular flow cytometry. The three stages were characterised by the differential expression of genes encoding many of the collagens and transcription factors related to articular cartilage, such as *COL2A1*, *COL9A1*, and *COL11A1*, *SOX9*, *SOX5*, and *SOX6*. The miRNA profiling revealed four clusters of expression patterns. One cluster consisted of miR-451, which was only upregulated in stage 1. Four miRNAs, including miR-140-3p, were upregulated in COL2A1 producing cells (stage 1 and 2). MiR-140-5p showed the same expression pattern and was significant with $p<0.05$. Another cluster consisted of five miRNAs, including miR-221 and miR- 222, that were upregulated in proliferating cells (stage 2 and 3). The last cluster consisted of 11 miRNAs, including miR-143 and miR-145, and was upregulated only in dedifferentiated cells (stage 3). Several of these miRNAs were predicted to regulate cartilage-related genes, such as *COL2A1, SOX9, SOX5* and *SOX6*. Because adult chondroblast-like cells (stage 2) still express type II collagen and other important cartilage-

related genes and miRNAs, they may be strong candidates for the treatment of articular cartilage lesions. The results from paper I formed the basis of papers II and III.

Paper II

In paper II, the global miRNA expression profiles of dedifferentiating ACs and differentiating MSCs were compared. The first comparison was between uncultured ACs and chondrogenically differentiated MSCs. MiR-140-5p and miR-140-3p were found to be among the most highly expressed miRNAs in both uncultured ACs and differentiated MSCs. In the next comparison, we identified miRNAs that showed a reciprocal relationship during dedifferentiation of ACs and chondrogenic differentiation of MSCs. The expression of miR-140-5p and miR-140-3p also changed the most during the dedifferentiation of ACs (downregulated) and the chondrogenic differentiation of MSCs (upregulated). Consequently, overexpression and inhibition studies of miR-140-5p and miR-140-3p were performed to investigate the function of these miRNAs.

Global mRNA analysis of transiently transfected and stably transduced cells showed that miR-140-5p and miR-140-3p upregulated genes associated with the ECM and downregulated genes associated with the cell cycle and the cytoskeleton. Further, chondrogenesis was impaired in MSCs stably overexpressing anti-miR-140-5p. This result was also supported by reduced GAG secretion and reduced expression of key chondrogenic markers as determined by western blot. Although many ECM-associated genes were downregulated when miR-140-5p was inhibited, the levels of *SOX9* and *ACAN* (aggrecan) mRNA were more or less unchanged. However, the protein levels were greatly reduced, showing that miR-140-5p positively regulates *SOX9* and *ACAN* post-transcriptionally. Thus, the dramatic effect of miR-140 on cartilage development both in vivo and in vitro may be explained by an unknown in which miR-140 enhances SOX9 and ACAN protein levels. This may occur either via a direct mechanism leading to increased translation or via an indirect

mechanism where miR-140 targets a gene responsible for inhibition of translation or the degradation of the proteins in question. In addition, several possible new targets of miR-140-5p and miR-140-3p were identified, including RALA, a small GTPase involved in TGF-β/Activin signalling. RALA was also verified to be a target of miR-140-5p.

Paper III

Based on results reported in paper I, we wanted to investigate the function of some of the miRNAs that were differently expressed during in vitro culture of ACs. These included miR-145, miR-140-3p and miR-140-5p. Surprisingly, our pilot experiments showed that a synthetic version of miR-145 (smiR-145), but not smiR-140-3p or smiR-140-5p, induced a strong immune response in both ACs and MSCs when transiently transfected with liposomes. Thus, it was necessary to establish a transfection method that did not induce immunological off-target effects for future experiments. In paper III, we report on the immunological off-target effects observed following liposome transfection of smiR-145 into human MSCs and human ACs. It was also demonstrated that the immunological off-target effect was mediated by the cytosolic dsRNA receptor RIG-I. The immune response was dependent on liposome delivery, as electroporation of smiR-145 did not induce an immune response. The dependency of RIG-I activation on liposome delivery has not been described before, and this finding opens new lines of investigation into RIG-I biology. Interestingly, an immune response, albeit at lower level, was observed by exposing the cells to liposomes only. In contrast to the smiR-145-induced immune response the liposome-induced immune response was not mediated by RIG-I. Clearly an immune response can potentially mask or change the cellular response to the synthetic miRNA that is used, making it very difficult to interpret the actual role of the miRNA under investigation. Based on these results, electroporation was the method of choice for transient transfection in paper II.

Methodological considerations

In this section, a brief introduction and some of the advantages and limitations of the methods included in this thesis will be presented. I will also mention some of the main obstacles encountered and which problem-solving strategies we performed to overcome these problems.

Cell culture

All ACs and MSC donors were isolated and cultured according to standard protocols described in the materials and methods in papers I, II and III. When appropriate, dedifferentiation of ACs was demonstrated by the reduced expression of *COL2A1*. All MSC donors fulfilled the criteria for the definition of human MSCs as proposed by ISCT [(210)].

Serum supplement

Serum supplies the culture medium with factors such as fatty acids, hormones, cytokines and other proteins that are essential for the survival and growth of cells in culture. Proteins such as fibronectin are also important for attachment of the cells to the culture vessel surfaces. Foetal bovine serum (FBS) has long been used as a serum supplement for ACs, MSCs and other types of cells. However, animal-derived products may be a source of viruses, prions or zoonotic infections [(211)]. The development of antibodies against bovine proteins was also demonstrated in patients receiving cells cultured in FBS and may lead to the rejection of the cells [(212)]. If cultured cells are to be used in cellular therapy, all animal-derived products should ideally be excluded from cell cultures. Both allogeneic and autologous serum have been shown to support growth and differentiation of MSCs, and pooled human platelet lysate-rich plasma (hPLP) is widely used in protocols for culturing cells today [(213-215)]. During the last two years, our research group has humanised all cell culture procedures, and hPLP is now routinely used for research purposes. This procedure involved comparison between cells cultured in FBS and hPLP. The expression patterns of common cartilage-related genes and

miRNAs, including *COL1A1*, *COL2A1*, *SOX9*, miR-140-3p, miR-140-5p, miR-145 and miR-221, were determined and demonstrated to have similar characteristics when FBS and hPLP were used as serum supplement. These data are not included in this thesis, but the results from papers I (FBS) and III (FBS and hPLP) reflect these findings.

Alginate

As described in the section "In vitro chondrogenesis using three-dimensional scaffolds" (page 30), both ACs and MSCs can be cultured using a wide range of three-dimensional scaffolds. Alginate is a naturally occurring polysaccharide consisting of β-D-mannuronic acid (M) and α-L-guluronic acid (G) monomers that are distributed randomly or as repeating or alternating blocks of G and M [(216)]. Divalent cations such as Ca^{2+}, Sr^{2+} and Br^{2+} interact with G-blocks and form three-dimensional gels in which the cells are encapsulated. The gels are easily dissolved in the presence of chelating agents such as citrate or EDTA [(216)]. Alginate has long been used for cell encapsulation purposes and maintains the chondrogenic phenotype of ACs in long-term cultures and promoting the re-expression of cartilage-specific genes in dedifferentiated ACs [(217,218)]. In our laboratory, the procedures for culturing and differentiating MSCs in alginate have been carefully characterised, and alginate was therefore used as a scaffold to support in vitro chondrogenesis of MSCs in paper II [(219-221)].

Transfection: overexpression and knockdown

Transfection is the procedure of introducing nucleic acids into cells, and it is used for overexpressing and knocking down genes and miRNAs. Commonly used transfection methods can be classified into three groups: 1) viral or biological methods (e.g., retroviruses and lentiviruses), 2) physical non-viral methods (e.g., electroporation and microinjection) and

3) chemical methods (e.g., the use of carriers such as lipids or calcium-phosphate) [222]. It is common to distinguish between stable and transient transfection. Stable transfection refers to cells that have integrated foreign DNA into their genome. The genes are therefore stably expressed even after replication. Transient transfection on the other hand, does not lead to integration into the genome. The genetic material is diluted during cell division and susceptible to degradation. Thus, the genetic material is only transiently expressed. Not all transfection methods are suitable for all cells, and huge variations in viability, transfection efficiency, toxicity, off-target effects and gene expression levels can be observed with different transfection methods. Transfection can also be highly affected by factors such as the culture medium, confluency, number of passages, the cell cycle and the quality of the genetic material used (Transfection methods overview, www.bio-rad.com/transfection). Below, some of the pros and cons of the transfection methods used in this thesis are briefly reviewed.

Liposomal transfection

During transient transfection using lipid-based methods, the lipid reagents are mixed with the nucleic acids. The lipids will then spontaneously form small vesicles (liposomes) containing the nucleic acids. Liposomes are thought to be taken up by endocytosis and transported via the endosomal pathway before releasing the nucleic acids into the cytoplasm. Fusion with the plasma membrane and direct delivery of the nucleic acids into the cytoplasm may also occur [182,183]. Some of the advantages of liposome transfection are that it is easy to perform, it is not very expensive, and it is highly efficient in certain cell types. However, some cell types are very difficult to transfect with these reagents, and the reagents are toxic at high concentrations. As already mentioned on page 37, liposomal transfection reagents may induce unwanted activation of the immune system, which can potentially mask or change the cellular response to the nucleic acids used for transfection. Lipofectamine 2000 and siPORT Transfection

Agent were used for liposomal transfection in paper III. Due to activation of immune responses a lot of time was used for investigating this phenomenon. In the beginning we could not exclude the possibility that the immune response was a result of smiR-145 regulating target genes involved in for example innate immune responses. However, after reading extensively in the literature and after performing many experiments we finally found a way to show that the immune response was dependent on liposomal delivery. When smiR-145 was delivered by electroporation no immune response was observed. Investigating immunological off-target effects was not one of our aims when this thesis was planned. It was an unexpected finding, but since the results were important for obtaining reliable data and because we had performed so many experiments to establish a transfection method that did not induce immune responses we decided to investigate this phenomenon in more detail. This was the basis for paper III and also the reason for using electroporation for transient transfection in paper II.

Electroporation

Electroporation is a method that uses electric pulses to make transient pores in the cell membrane enabling nucleic acids to be transported across the cell membrane, directly into the cytosol without involving any active transport mechanisms [(223)]. This method is highly efficient and can be used on a wide range of cells. Additionally, it is easy to perform. On the other hand, electroporation often results in high cell mortality. This was the main problem when optimizing the electroporation procedure. In order to solve this problem several experiments were performed. It turned out that the amount of serum used was crucial for survival. In our initial experiments culture medium containing 10% FBS was used. However, when switching to 20% FBS most cells survived. The number of cells used for each reaction also seemed to matter. $1x10^6$ cells per reaction was optimal in our experiments. Higher cell mortality was observed when using 500.000 cells per reaction. The reason for this is not

known, but cell membranes may function as resistance to the electrical current used for electroporation. By reducing the number of cells, each cell is exposed to a higher electrical current (current was kept constant), which presumably contribute to cell mortality. On the other hand, using more than $1x10^6$ cells per reaction the transfection efficiency was reduced. Although 20% hPLP was used in some electroporation experiments (paper II) there seem to be no difference in viability between 10% or 20% hPLP. The reason for this is unknown. The Nucleofection technology from Lonza (an electroporation technique in which nucleic acids are transferred into both the cytoplasm and nucleus) was used in papers II and III.

Lentiviral transduction

Dedifferentiation of ACs and differentiation of MSCs often involves culture periods of two to three weeks. Because the effect of transient transfection only last a few days, we decided to use stable transfection to investigate the function of miRNAs during these two processes. A lentiviral transduction system that utilises the third generation of replication incompetent HIV-based expression vectors was used for this purpose (paper II) [(224)]. Briefly, the process involves transient transfection of separate viral constructs encoding all the proteins needed for production of viral particles and an expression vector encoding "the gene of interest" into a so-called packaging cell line. The expression vector is then packaged into viral particles and secreted into the supernatant by the packaging cells. The viral particles (supernatant) are then harvested and transferred to the desired target cell, resulting in infection, reverse transcription of the expression vector and integration into the genome of the target cells. The main advantage of lentiviral transduction is that both dividing and non-dividing cells can be transduced with high efficiency, resulting in sustainable transgene expression. However, lentiviral transduction is a time-consuming process, and because the viral vectors are integrated randomly into the genome, there is a risk of disrupting tumour suppressor genes.

Third-generation replication-incompetent HIV-based expression vectors have been modified in several ways to enhance their biosafety, and they are considered biologically safe. However, because these vectors can transduce human cells, there is always a risk for the investigators. Special laboratory facilities and practices that are in line with established health and safety guidelines are therefore required for experiments utilising lentiviral vectors. The main obstacle in optimising transduction seemed to be induction of senescence in the MSCs. The cells did not proliferate more than one passage after lentiviral transduction in our initial pilot experiments. In an attempt to solve this problem, serum source, cell number, different MOI (Multiplicity of Infection) and several concentrations of transduction reagents was investigated. Transduction using hPLP as serum supplement resulted in much lower transduction efficiency compared to FBS in all experiments. Thus, incubation with viral particles overnight was performed using FBS as serum supplement. Polybrene and puromycin are often used in transduction experiments to increase transduction efficiency and as a selection antibiotic for transduced cells, respectively. High doses of both polybrene and puromycin seemed to induce senescence in the MSCs in our pilot experiments. By reducing the amount of polybrene and puromycin and increasing the MOI we were able to transduce MSCs with high efficiency and culture the cells for at least two to three passages after transduction. The transduced cells were also successfully differentiated into chondrocytes for three weeks and adipocytes and osteocytes for four weeks. The MOI and concentration of transduction reagents used in paper II were based on results from pilot experiments performed over several weeks. Compared to MSCs, ACs did not undergo senescence in any of the experiments. However, the same transduction conditions were used for both cell types in paper II.

Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR)

RT-qPCR is one of the most powerful methods for quantification of RNA due to its high specificity, sensitivity and reproducibility. The process of RT-qPCR involves four steps: (1) preparation of RNA, (2) reverse transcription (RT) to cDNA, (3) amplification of cDNA by PCR and (4) data analysis [225]. During the first PCR cycles, the cDNA template is doubled, resulting in exponential amplification. Thereafter, primarily because of reagent limitations, the PCR reaction slows down and the PCR product is no longer doubled at each cycle. In the exponential phase, the PCR products formed are directly proportional to the amount of RNA template in the starting material [226]. RT-qPCR measures the amount of PCR products formed in the exponential phase, making it possible to determine the initial amount of target RNA in the starting material. The detection and quantification is possible because of fluorescent dyes or probes that bind to the PCR products formed after each PCR cycle. The intensity of the fluorescent signal is detected by the RT-qPCR machine and reflects the amount of PCR product formed. For the quantification of mRNA and miRNA, two methods can be performed: absolute quantification (AQ) and relative quantification (RQ). AQ determines the exact number of target RNA molecules present in the starting material by comparison with standard curves [226]. On the other hand, RQ uses mathematical equations and determines the change in expression relative to a reference sample, such as an untreated sample, without requiring the exact number of target RNA molecules [226]. In this thesis, RQ was used for all RT-qPCR analyses. There are certain requirements for the RQ data to be valid. First, it is important that the amount of cDNA formed in the RT reaction reflects the amount of RNA input [227]. If too much RNA is used in the RT reaction, not all RNA will be reverse transcribed to cDNA due to a lack of reagents or the presence of inhibitors within the sample. This may lead to false values. This can be tested by preparing a dilution series of the RNA [227].One of the first pilot

experiments I performed in our laboratory was to test this using five different concentrations of RNA input. Not in any of the experiments did I observe any problems related to RNA input. We do not test this in every single experiment performed in our laboratory, but the pilot experiments indicate that this is not a common problem in the experimental settings used in this thesis. Second, for normalisation of RT-qPCR data, an internal reference gene is amplified simultaneously as the target gene. The expression level of the reference gene has a huge impact on the results, and it should ideally be expressed at constant levels in all samples investigated. Therefore, it is highly recommended that reference genes be validated for each experiment to ensure constant expression within all samples used for comparisons [(228)]. This was an issue in paper I where uncultured ACs had very different expression of typically used internal reference genes compared to cultured ACs. Actually we had to test thirty-two different internal reference genes before we found one that was expressed at similar levels in both uncultured and cultured ACs. Interestingly, we have observed exactly the same in uncultured and cultured human endothelial cells. Third, the RQ method is only valid if the amplification efficiency of both the target gene and the reference gene is approximately equal (above 90%). This can easily be assessed by diluting the RNA from each sample and looking at how the results vary relative to the dilution, as previously described [(226)]. In our RT-qPCR analysis we have used Taqman gene expression assays. These assays have been validated by the manufacturer to have the same amplification efficiency. However, we have checked the amplification efficiency of some of the assays we routinely use in our experiments using the method described by Arya et al. [(226)]. All accept one showed similar amplification efficiency. This assay on the other hand was not used in this thesis. RT-qPCR analysis is fast, and when properly performed, it is one of the most sensitive and powerful methods for RNA quantification. Because gene expression can be regulated at many steps, from the genome (genes) to the final protein product [(12)], the major drawback of RT-qPCR and other RNA

quantification methods is that RNA levels do not always reflect the corresponding protein levels [229]. In these situations, RNA quantification is not always useful.

Global mRNA and miRNA analysis

Global gene expression analysis is one of the most widely used methods in biology today, and it provides quantitative information about the transcriptome of cells [230]. Both mRNA and miRNA transcriptome profiling were performed in this thesis. In this type of approach, most genes and miRNAs are represented as oligonucleotides on a small array. The samples are usually reverse transcribed to cDNA or cRNA and labelled with biotin (or a fluorescent dye) and applied to the array. Because of the hybridisation properties of nucleic acids, the labelled nucleic acids will bind to their complementary strands on the array. After washing away non-hybridised material, the arrays are stained with a streptavidin-Cy3 conjugate (streptavidin binds biotin and Cy3 emit fluorescence). After another washing step, the array is placed in a scanner that measures the amount of fluorescent signal created. The intensity of the fluorescent signal is proportional to the amount of mRNA/miRNA in the original sample. One of the main advantages of transcriptome profiling analysis is that thousands of genes can be studied simultaneously, making it possible to identify new genes that are important for different biological processes. It is also relatively cheap and quite fast. However, there are many possible sources that can contribute to errors in microarray analysis, such as sample collection, labelling, hybridisation efficiency and fluctuation in scanning the fluorescent signal [231]. Transcriptome profiling provides huge data sets that need to be analysed carefully. Several statistical and data analysis tools are available for this purpose. It is important to use these tools carefully and keep the sources of variation in mind when processing the data. As with RT-qPCR, transcriptome profiling only provides quantitative data for RNA molecules and not their encoded proteins. The Norwegian Microarray Consortium performed several

quality controls on our microarray data as described in paper II and III. In this thesis the J-express software was used for analysing microarray data. The software is user friendly and is free of charge. Several members of our group, including me, have attended a course to learn how to use this software. Microarray analysis software can be used to analyse data in several ways using different methods and statistical algorithms. For our studies we chose to make simple gene lists of differently expressed genes and gene ontology analysis in order to classify the genes according to functionality. To ensure that our analysis was correctly performed we also went through our experimental strategy and analysis together with a bioinformatician from The Norwegian Microarray Consortium.

Flow cytometry

Flow cytometry is used to measure the fluorescence and optical properties of cells (and other particles) contained in a single-cell suspension. Within a stream of fluid, single cells flow through the beam of a laser. Light is then emitted and scattered in all directions. The forward scattered light gives information about cell size, while the side scattered light gives information about the intracellular granularity of the cells. With the use of fluorescent dyes or fluorescent conjugated-antibodies, it is possible to measure the DNA and RNA content, enzyme activity, membrane potential, intracellular calcium flux, the levels of intracellular proteins and proteins expressed on the cell surface [232]. Flow cytometry analysis is rapid and provides quantitative data. Another advantage is that it is possible to study subpopulations within a heterogeneous cell population. The principles of flow cytometry can also be exploited to sort and isolate cells with great precision. This technique is called fluorescence activated cell sorting (FACS). Although flow cytometry can be used for many different applications, it also has some limitations. Flow cytometry requires cells to be in suspension, meaning that cells inside tissues have to be released in order to be analysed. The software and

the analysis can be quite sophisticated, and advanced analysis requires highly skilled operators. It is also important to ensure the specificity of the antibodies used to avoid nonspecific binding to other proteins. This can in some situations be checked by western blot analysis [233]). Flow cytometers are also quite expensive. The experimental settings used for flow cytometry in this thesis were straight forward, and I did not run into problems in these analyses. However, sorting of cells in paper II was performed by The Flow Cytometry Core Facility at Oslo University Hospital as we had little experience with this procedure.

Multiplex bead array assay

Multiplex bead array assay analysis is based on many of the same principles as flow cytometry (fluidics, laser, optics and the use of antibodies), and it can be used to detect many different proteins in the same sample at the same time. Small beads of two colours and with different intensities are coated with capture antibodies against specific proteins and then mixed with the sample (such as cell culture supernatants, serum or cell lysates). The protein bound by the capture antibody is then detected using a fluorescent-coupled reporter antibody. Because of the different colour intensities of the beads, each bead coupled with a specific capture antibody can be identified, while the signal intensity from the fluorescent-coupled reporter antibody is used for quantification of the protein. The combination of these two features makes it possible to measure 100 proteins in the same sample. Two lasers are used for this purpose. Multiplex bead array is a quantitative method, and it uses very small sample volumes. For large-scale analysis, this approach will reduce both the cost and time required compared to enzyme linked immunosorbent assay (ELISA), which has been the standard method for quantitative analysis of cytokines and other proteins. Although it has been shown to be comparable with the widely used ELISA [234], the sensitivity of bead assays from different suppliers has been questioned [235]. In this thesis multiplex bead array assay was

performed as a small part of paper III. Both the experimental procedure and the data analysis were performed under supervision of Julie Katrine Lindstad who is a highly experienced user of this system.

Western blot (immunoblot)

Western blot is a widely used method that utilises antibodies for the detection of proteins in tissue samples, serum, cell lysates or cell culture supernatants. The technique involves separation of the proteins according to their size by electrophoresis and transfer of the separated proteins onto a polymer membrane using an electrical current. A primary antibody against the protein of interest is then added to the membrane. After washing away unbound primary antibodies, a labelled secondary antibody is added. The labelled secondary antibody recognises the primary antibody, which is bound to the protein, and it emits a signal that can be detected. This is a typical set-up, but there are other set-ups as well (Alegria 2009). The signal developed depends on the labelling technique. The labelling approach may involve radioactivity, chemiluminescence, fluorescence or colloidal gold. The signals are developed using X-ray film or detection machines that create a digital image of the signals. Traditional western blots are considered to be semi-quantitative, but by use of proper controls, this method can be used for quantitative analysis [(236)]. Western blotting has a very high specificity and sensitivity. However, the procedure involves many steps and reagents, and each step has to be optimised for successful detection and identification of the desired protein. The major obstacle with western blot analysis in this thesis was to extract proteins from cells cultured in alginate discs. Releasing the cells from the alginate disc using sodium citrate or EDTA was not successful, as most cells were lost during the procedure. Several other procedures were tried without success, including pulverising the alginate disc in liquid nitrogen and extraction of proteins using the Trizol method. This method included precipitation and solubilisation of

the proteins. However, we were not able to solubilise the precipitated proteins. At the end it turned out that a relatively simple procedure of pulverising the alginate disc in liquid nitrogen and resuspending the cell/alginate powder directly in the loading buffer (laemmli) worked very well. Still, the western blot bands obtained from cells cultured in alginate were less distinct than from cells in monolayer cultures, particularly ACAN bands.

Immunohistochemistry/immunofluorescence

Immunohistochemistry (IHC) is a technique that utilises antibodies to investigate the localisation and distribution of proteins (or other structures) in tissue samples. IHC is performed by using either fluorescence-coupled or enzyme-coupled antibodies. The principle is the same for both methods. The procedure involves fixation and sectioning of the tissue sample, incubation with primary antibodies, washing away unbound antibodies, incubation with secondary antibodies, washing and development of the signal. A fluorescence microscope is used to both detect the signal and to determine the localisation of the protein. Most fluorescent microscopes contain filters that make it possible to visualise different colours. By using secondary antibodies coupled with fluorochromes with different emission spectra, it is possible to visualise different proteins in the same cell. The ability to determine the exact location of specific proteins in the cell is the major advantage of IHC. The major limitation is the potential for non-specific binding of the antibodies. Therefore, antibody specificity should be validated before use (for example by western blotting). Similarly to western blotting, the IHC procedure involves many steps and each step has to be optimised for successful detection. Fixation of the tissue is the most critical step in the procedure. Fixation is performed to conserve the architecture, or morphology of the tissue, but unsuccessful fixation may result in artificial staining patterns. Thus, optimisation using several fixation reagents is recommended. Extensive optimisation of this procedure on

alginate discs and validation of all antibodies used in this thesis were performed over a period of several months. This work was performed by a former colleague, Axel Küchler, who has great knowledge and experience with this technique.

MiRNA luciferase reporter assay

Today, the only method available to validate an mRNA sequence as a binding site for a specific miRNA is the use of reporter genes containing the potential binding site [237]. MiRNAs bind to complementary sequences within the mRNA (most often the 3'UTR), resulting in degradation of the mRNA or translational inhibition. To validate an mRNA sequence as a miRNA target, the 3'UTR of the mRNA (or other potential target sequences) can be cloned into a plasmid constitutively expressing luciferase, which emits light. When this construct is co-transfected together with a miRNA mimic, the light output will be reduced if there is a binding of the miRNA mimic to the 3'UTR. A commercially available reporter assay was used to validate the targets of miR-140-5p in paper II. These commercial kits are quite expensive, but they reduce the amount of work required because the 3'UTR is already cloned in the luciferase plasmids. The procedure is very fast and easy to perform. We did not experience any problems using this assay.

Quantification of sulfated proteoglycans and glycosaminoglycans (GAGs)

In paper II, the sulfated proteoglycans and GAGs present in the culture medium of chondrogenically differentiated MSCs were quantitated using a commercial kit. The kit is based on a quantitative method in which a blue dye (Blyscan) turns pink when it binds to sulfated GAGs. By comparing the unknown concentration in the sample with a calibration curve made of standards with a known concentration, the amount of sulfated proteoglycans

and GAGs in the medium can be determined. This is a standard method used for this purpose, and it has been used in our laboratory for several years. The removal of insoluble materials, such as ECM molecules or cell debris, is crucial for obtaining reliable results, as these materials can interfere with the assay. It is a very fast, easy and robust procedure. No troubleshooting was required in these analyses.

General discussion

Articular cartilage is a highly specialised tissue with poor healing capacity. Injury to the articular cartilage results in pain and stiffness of the joints and strongly affects the quality of life for many people worldwide. Several surgical strategies have been developed and are used to treat patients today, but the repair tissue does not consist of hyaline cartilage; rather it is fibrocartilage or a mix of fibrocartilage and hyaline cartilage [52]. This repair tissue does not have the properties of hyaline cartilage and often degrades over time [44]. Thus, the goal in the field of cartilage repair is to develop a treatment that results in the formation of perfect hyaline cartilage that integrates with the surroundings of the lesion and last for the rest of the patient's life. ACI, one of the most widely used cell-based treatments for cartilage lesions, involves isolation and monolayer expansion of autologous ACs [53]. A major limitation with ACI is that monolayer expansion of the ACs result in the dedifferentiation and loss of the hyaline cartilage phenotype [56,57]. Avoiding dedifferentiation may be crucial for improving ACI. Another strategy is to use MSCs that have the capability for chondrogenic differentiation [90]. However, there were no differences in the clinical outcomes between patients receiving MSCs or ACs for ACI, suggesting that both approaches result in the formation of a similar repair tissue [238]. Understanding the molecular mechanisms leading to dedifferentiation and chondrogenic differentiation may result in new strategies to improve the clinical outcome of cartilage repair.

Papers I and II

When this thesis was begun, our group had recently published a paper showing that ACs cultured within their own ECM synthesised type II collagen for approximately two weeks before starting to spread out onto the culture surface and dedifferentiate [239]. Inspired by these

results, we decided to characterise these cells in more detail. At that time, not many studies had investigated the role of miRNAs in cartilage, and no studies investigating changes in miRNA expression during in vitro culture of ACs were available. As miRNAs are known to regulate many cellular processes, we wanted to investigate the changes in miRNA expression occurring during in vitro expansion of ACs.

In paper I, uncultured ACs were established in culture and expanded as previously described [239]. We then investigated the cells at different time points with respect to their expression of a large number of genes, miRNAs and proteins. As described in paper I, the ACs went through three stages during 28 days of in vitro culture: (1) primary, uncultured ACs (day 0); (2) the chondroblast-like stage (day 7–14); and (3) the dedifferentiation stage at the end of the culture. In most ACI procedures, ACs are expanded as monolayer cultures from the day of isolation, resulting in implantation of dedifferentiated ACs [52,240]. By expanding the ACs in their own ECM, sufficient numbers of cells required for ACI are obtained within two weeks [239]. These cells are not dedifferentiated, as they express high levels of several collagens and transcription factors related to hyaline cartilage (paper I, Figure 1). Compared to fully dedifferentiated ACs, implantation of chondroblast-like cells may lead to a repair tissue that is more similar to hyaline cartilage. Today, ACs to be used for ACI are cultured in this way in our GMP laboratory. Notably, during the development of OA, the degradation of type II collagen and aggrecan are accompanied by a phenotypical change in which the ACs express molecules associated with matrix mineralisation, including type X collagen [241]. Thus, matrix degradation seems to trigger hypertrophic differentiation. In addition, the synthesis of type II collagen and aggrecan are also observed during early OA, perhaps to compensate for the loss of these molecules, but the proteins are often degraded or damaged [242-244]. This situation resembles some of the gene expression changes observed in paper I (Figure 1). When ACs are harvested and isolated for ACI, the cells are enzymatically released from the

cartilage biopsy. This obviously involves degradation of the matrix. Perhaps ACs released from the matrix are stimulated so that hypertrophic differentiation is set to be the default pathway. If so, it is possible that chondroblast-like cells will not be any more effective than fully dedifferentiated ACs. This possibility was not investigated in this thesis and needs to be validated in in vitro and clinical trials.

As miRNAs were likely to be differently expressed during in vitro culture, a global miRNA analysis was performed in cells at each of the three stages. Several miRNAs were downregulated during culture of ACs, including miR-30d, miR-210, miR-451 and miR-563 (paper I). The miR-30 family has been shown to target *RUNX2* and *SMAD1* and negatively regulate osteoblast differentiation [(245)]. The function of miR-210 and miR-563 in cartilage development is unknown. However, miR-210 is considered to be the major hypoxia-inducible miRNA [(246)] and is upregulated during chondrogenesis both in vivo and in vitro [(168)] (paper II). miR-210 also promotes osteoblast differentiation by inhibiting TGF-β/activin signalling [(247)]. Nevertheless, as ACs experience hypoxic conditions in vivo, it is reasonable to suggest an important role of miR-210 in cartilage biology.

Some of the miRNAs that were upregulated during the dedifferentiation of ACs included miR-132, miR-138, miR-145, miR-221 and miR-222. We also reported the possible targets of several of these miRNAs, including *SOX9* as a target of miR-145 and *SOX5* and *SOX6* as targets for miR-132 and miR-138, respectively. Since then *SOX9* has been verified as a target of miR-145 in two studies [(162,163)] In bovine articular cartilage, miR-222 plays a role in mechanotransduction [(164)], while miR-221 has been reported to be a negative regulator of chondrogenesis in chick limb mesenchymal cells [(165)]. In paper I, miR-140-3p and miR-140-5p were reported to follow the expression pattern of *SOX9* (downregulated during dedifferentiation). At that time, miR-140 was already known to be cartilage-specific and important for the development of pharyngeal cartilage in zebrafish. Indications also suggested

that SOX9 regulated the expression of miR-140, which was in line with our findings [151,152,248]. During our preparation of paper I, two articles on miR-140 were published by the same group [156,249]. The first paper compared miRNA expression in ACs with that of undifferentiated hMSCs and found miR-140 to have the largest difference in expression between the two cell types. In addition, miR-140 was reduced in OA compared with healthy articular cartilage and was downregulated by the OA-associated cytokine IL-1β [249]. The second paper demonstrated an important role for miR-140 in cartilage homeostasis and cartilage development as described on page 34. Briefly, miR-140 knockout mice were shown to have a shorter skeleton and an OA-like pathology, and ADAMTS5, an aggrecanase, was validated as a target of miR-140 [156]. Immediately following the publication of paper I, SOX9 was confirmed to positively regulate miR-140 transcription [153]. Clearly, miR-140 had a profound effect on cartilage development, but the main mechanism had not been demonstrated. In paper II, we compared the miRNA expression profiles of chondrogenically differentiated MSCs and uncultured ACs. MiR-140-5p and miR-140-3p were among the most highly expressed microRNAs in both differentiated MSCs and uncultured ACs, and their expression changed the most during culture (paper II, Figure 1, Table 1 and Supplementary Table S4). At the global mRNA level, several genes encoding cartilage/ECM molecules were downregulated after inhibition of miR-140-5p, while genes associated with the cell cycle and cytoskeletal remodelling were upregulated. Further, inhibition of miR-140-5p inhibited GAG synthesis in differentiating MSCs and SOX9 and ACAN were downregulated at the protein level, although no consistent differences were observed at the mRNA level. It is well known that miRNAs may directly regulate gene expression at the translational/protein level [250-252]. In paper II we found potential binding sites for miR-140-5p in the 5'-UTR of both *SOX9* and *ACAN* mRNA. miR binding to the 5'-UTR has previously been associated with translational enhancement [251,252]. Thus, the positive post-transcriptional regulation of these molecules may occur via a direct

mechanism leading to increased translation. Another possibility is an indirect mechanism where miR-140 targets a gene responsible for inhibition of translation or the degradation of the proteins in question. This result demonstrates how important it is to analyse protein levels when investigating miRNAs. In many situations, there is no correlation between the mRNA and protein levels [229]. Microarray analysis may be useful as a screening tool to look for miRNA targets and assess the global effect after manipulation of miRNAs. However, important regulatory molecules may be overlooked, and it is highly recommended to at least investigate protein levels for key regulators of the processes being studied. The huge effect on SOX9 and ACAN proteins and chondrogenesis was perhaps not obvious compared to the levels of miR-140-5p inhibition after anti-miR-140-5p treatment (paper II, Figure 5a). Notably, the lentiviral vector used for inhibition of miR-140-5p produces an RNA that is fully complementary to endogenous miR-140-5p. The complementary RNA binds the endogenous miR-140-5p and forms a thermodynamically stable duplex and thereby inhibits its function by preventing miR-140-5p from binding its targets. The anti-miR molecules are not expected to degrade endogenous miRNA levels. Thus, the reduced miR-140-5p levels measured after inhibition also included the bound and inhibited miR-140-5p and are therefore likely to be an underestimation. The reduced levels of miR-140-5p in paper II (Figure 2B and Figure 5A) are most likely a result of reduced transcription due to the reduced SOX9 protein levels and not a result of degradation. This is also supported by the fact that miR-140-3p (which should not be a target of anti-miR-140-5p) is reduced to more or less the same levels as miR-140-5p (paper II, Figure 2B)

The data from paper II support the results from earlier in vivo studies in which miR-140 protects against proteoglycan loss and the development of OA [156]. Not surprisingly, miR-140 is downregulated in osteoarthritic cartilage [172,249]. Recently, reduced levels of miR-140 were also detected in the synovial fluid of OA patients compared to control samples [253]. As miR-

140 is regarded as a tissue-specific miRNA and has such a dramatic effect on cartilage homeostasis, it has the potential to serve as a biomarker for the development of early OA. MiR-140 can also be detected in the plasma and other tissues beside cartilage, showing that its expression is not restricted to cartilage tissue [254,255]. Regardless, if miR-140 from the synovial fluid or plasma can be used as a biomarker to predict early OA, it would provide a very easy and cheap method that could improve treatment and be of great help in the effort to understand the underlying causes of OA [256].

Apart from miR-140-5p and miR-140-3p, several other miRNAs were differently expressed between uncultured ACs and differentiated MSCs (paper II). Among the top 100 expressed miRNA between the two cell types, 57% were common to both cell types. Thus, many of the miRNAs expressed or induced by in vitro chondrogenesis may in fact be undesirable as they are not expressed in uncultured ACs. These miRNAs may even be responsible for the expression of proteins such as type I and type X collagen that are present during chondrogenic differentiation of MSCs [220]. One example is miR-181a, which promotes type X expression in pre-hypertrophic/hypertrophic chondrocytes (oral presentation: IADR General Session, Barcelona, Spain, July 14-17, 2010). MiR-181a was barely detected in uncultured ACs, but it was highly upregulated during chondrogenic differentiation of MSCs (paper II). One strategy to improve chondrogenesis for tissue engineering purposes may be to inhibit miRNAs that are not expressed in native cartilage but highly expressed in the differentiating cells or to overexpress miRNAs that are highly expressed in native cartilage but not expressed in differentiating cells. As shown in paper II, one miRNA can regulate hundreds of genes, demonstrating the potential use of miRNAs in therapeutics and cartilage repair. Another strategy is to manipulate more than one miRNA. By overexpressing the miR-302/367 family, Anokye-Danso et al. showed that somatic cells could be reprogrammed into iPSCs with similar characteristics as ESCs, including germline and chimera contributions [257].

These findings demonstrate the powerful effect of miRNAs. Manipulating only a few miRNAs enabled cells to completely change their epigenetic landscape and transcriptome and in essence become another type of cell. Perhaps manipulating only one or a few miRNAs will aid in developing cartilage cell therapies that eventually relieve the suffering of millions of people.

Paper III

Paper III is not directly related to chondrogenesis and dedifferentiation but is more concerned with the methods used for investigating miRNA functionality. These results were very important for us to continue our work to investigate the function miRNAs.

As already discussed in paper I, miR-145 was highly upregulated during dedifferentiation and showed an inverse relationship with *SOX9* expression. Bioinformatic analysis also identified *SOX9* as a potential target of miR-145 (paper I). Based on our own findings and published data, we decided to continue our investigation of several miRNAs, including miR-145. To identify targets and unravel the molecular mechanisms of chondrocyte dedifferentiation, we subsequently performed pilot experiments in which liposome-mediated transient transfection was used to overexpress and inhibit miRNAs in ACs and MSCs. Surprisingly, a strong immune response against a synthetic miR-145 mimic (smiR-145) was observed, but not against any other sequences tested. Previously, it was shown that siRNAs (very similar to synthetic miRNAs) induced immune responses through TLRs located in endosomes [(192)]. Understanding the mechanisms behind these responses and avoiding them is important for obtaining reliable data that are specific to the microRNA investigated. Clearly, liposome transfection could lead to misleading results. For example, siRNAs against VEGF were shown to inhibit vascularisation in mice [(258)]. However, it was demonstrated that

different control siRNAs targeting non-mammalian and non-expressed genes had the same effect due to stimulation of interferons [259].

We therefore decided to investigate this phenomenon in more detail and to see if it was possible to avoid the immune response. Thus, the objective of paper III was not to investigate the specific role of miR-145 as a miRNA, but rather to understand its immunological off-target effects. As shown in paper III, the smiR-145-induced immune response was mediated by RIG-I and was dependent on liposomal delivery, as electroporation of smiR-145 did not lead to an immune response in either MSCs or ACs. Based on these results, we decided to use electroporation for transient transfection in paper II.

Perhaps the most surprising result in paper III is that RIG-I required liposomal delivery for activation in certain situations. This has not been described before. It has been proposed that once foreign RNA is located in the cytoplasm, it will be recognised by RIG-I independently of the delivery mechanism and immediately induce an immune response as a defence mechanism against viruses. Liposomal delivery utilises the same pathways as many viruses for entering cells [182-184,260]. There are two main routes for virus entry. The first is the endocytic route in which viruses enter cells via endocytosis and are transported through the endosomal pathway. The other route is the non-endocytic route, which involves fusion with the plasma membrane and direct entry into the cytoplasm [261]. It is already known that endosomal TLRs respond to viruses entering the endosomal pathway [191]. Perhaps the role of RIG-I is to survey the actual entry point of the non-endocytic route instead of keeping the entire cytoplasm under surveillance as previously thought. This would be a much more efficient strategy. However, this hypothesis needs to be investigated further. In any case, the choice of delivery method does affect the ability of RIG-I to respond to certain RNA sequences. For researchers investigating RIG-I biology, this possibility should be of interest for further investigation.

It is well known that cells secrete small vesicles (exosomes) containing miRNA, mRNA and proteins that are taken up by neighbouring cells. MSCs express high levels of endogenous miR-145 (paper III, Supplementary figure S3), and they secrete exosomes [262]. In theory, exosomes containing endogenous miR-145 could activate an immune response via RIG-I when taken up by neighbouring cells. The sequence of smiR-145 is identical to the endogenous miR-145, and it could be speculated why MSCs do not persistently express immune genes when they express very high levels of endogenous miR-145 and if RIG-I reacts with self-RNA. Although it has been suggested that RIG-I is involved in sensing self-RNA [263], it is not likely to be the case with endogenous miR-145. First of all, untransfected MSCs do not express *CXCL10* (paper III, Figure 1). If exosomes induced an immune response, MSCs should constantly express *CXCL10*. Second, exosomes secreted by MSCs actually possess an immunosuppressive activity [262]. One explanation could be that these exosomes do not contain miR-145 and therefore do not induce an immune response. On the other hand, it is known that chemical modification of siRNAs inhibits immunological off-target effects (judge), and more than 100 natural chemical modifications of endogenous RNA have been described so far [264]. Although the sequences of smiR-145 and endogenous miR-145 are identical, they are definitely not identical with regards to chemical modification. Such naturally occurring modifications may indeed be used to distinguish self from non-self RNA [265]. In other words, if it was possible to transfect endogenous miR-145 using liposomes, RIG-I would probably not be activated by miR-145. In my opinion, this is a more likely explanation for the lack of a persistent immune response in MSCs. In paper III, 5'ppp and blunt ends versus 2'nucleotide overhangs were discussed as possible mechanisms for discriminating between self and non-self RNA. Natural chemical modification is yet another mechanism by which the cell can discriminate between self and non-self RNAs.

Immunological off-target effects may not only lead to biased results, but they also raise concerns about the safety of these reagents for potential use in clinical trials. However, in certain circumstances such as cancer therapy, viral infections and vaccine development, stimulating an immune response may be desirable. Interferons inhibit angiogenesis and have been used for the treatment of cancer for a long time [266]. Several studies have therefore explored the possibility of using immunostimulatory nucleic acids for cancer therapy [267]. TGF-β1 is elevated in tumours and is therefore a potential target in cancer therapy. Recently, Ellermeier et al. combined the silencing effect of siRNA against TGF-β1 and immune activation via RIG-I to study the effect in a mouse model of pancreatic cancer. By adding 5'ppp to the TGF-β1 siRNA, the mouse immune system was activated, and prolonged survival compared to mice that received an unmodified TGF-β1 siRNAs and a 5'ppp-control siRNA. Notably, both unmodified TGF-β1 siRNAs and a 5'ppp-control siRNA reduced tumour formation compared to unmodified control siRNA [268].

INF-β was one of the molecules that were highly expressed after liposomal transfection of smiR-145. It has been suggested that INF-β can be used for treatment of arthritis [269]. Thus, manipulating miRNAs involved in arthritis with immunostimulatory miRNA mimics or inhibitors could potentially be used in therapy using the same strategy as that described by Ellermeier et al.

Liposomes and immunostimulatory nucleic acids have also been explored and used as adjuvants for vaccine development [270-272]. In vaccine development, the goal is to induce an immune response against a specific antigen to provide long-term protection against infection. Adjuvants are substances that are used in combination with the antigen to enhance or improve the immune response to the antigen. As shown in paper III and elsewhere [184-186], liposomes induce an immune response, but liposomes alone are not sufficient to activate antigen-presenting cells, which are important for developing effective vaccines [271]. On the other hand,

activation of antigen-presenting cells can be achieved by stimulating PRRs [271,272]. The combination of liposomes with immunostimulatory smiR molecules may therefore be a strategy for improving vaccine development [271]. However, effective use of liposomes and immunostimulatory smiR molecules in therapy will require a detailed understanding of the immunological properties of each reagent [272]. Currently, it is difficult to predict which sequences will induce immune responses and how strong the response will be. Such responses will also vary between cell types, making this a complicated task. The recently established RNA immuno database provides an opportunity to analyse and compare the results of studies using RNA inhibition techniques and may be used as a guide to avoid or minimise immunological off-target effects, but it may also be useful for predicting immunological off-target effects in therapy [273]. The data in paper III may result in a better understanding of RIG-I biology in the future and will hopefully contribute to our understanding of how the manipulation of miRNAs can be safely used in clinical protocols.

The liposome-induced immune response reported in paper III was different from the smiR-145 response and included the production of inflammatory cytokines such as interleukin 6 (IL6), IL8 and IL-1β (paper III, Figure 8a,b). Others have also reported this phenomenon, and recently it was shown that such liposome-induced immune responses involved STING, an ER-resident transmembrane protein [184]. Although the receptor for the liposome-induced immune response has not been identified in our study, TLR4 or lipid receptors in the plasma membrane may be involved [188-190]. This observation may lead to a better understanding of the early events in the development of atherosclerosis. Atherosclerosis is considered as an inflammatory disease and results in the deposition of lipids in arteries and may eventually lead to blockage of the arteries and myocardial infarction [274]. At an early step in the process of atherosclerosis, endothelial cells are activated by lipids, resulting in secretion of inflammatory chemokines, including CCL5, CXCL10, IL6, IL8 and IL1β, which was also

upregulated by liposomes as shown in paper III (Figure 1, 2 and 8) [274-276]. In the atherosclerosis field, much attention has been focused on PRRs because cholesterol has been shown to upregulate IL1β, which is considered to be the gate-keeper of inflammation [277-279]. The mechanisms mediating these responses are not fully understood [274], but it is possible that physiological lipids induce an immune response in the same way that the liposomes in our experiments did. This is one of the follow-up studies that our group is planning to undertake.

Conclusions

1. Chondroblast-like cells expressed genes and synthesised proteins typical of hyaline cartilage. Whether these cells will produce better hyaline cartilage than dedifferentiated chondrocytes or MSCs needs to be tested in formal assays for in vitro chondrogenesis and, eventually, in clinical trials.

2. Several miRNAs were differently expressed during dedifferentiation of ACs. Many of the miRNAs were predicted to have key chondrogenic genes as targets.

3. Chondrogenically differentiated MSCs and uncultured ACs expressed many of the same miRNAs but were quite different at the global level. Furthermore, several miRNAs showed a reciprocal relationship during dedifferentiation of ACs and chondrogenic differentiation of MSCs.

4. MiR-140-5p had a profound impact on chondrogenesis and positively regulated SOX9 and ACAN post-transcriptionally via an unknown mechanism. Moreover, global mRNA analysis also indicates the involvement of miR-140-5p in cytoskeleton remodelling and cell division.

5. RALA was confirmed as a new target of miR-140-5p and may play an important role in cartilage biology.

6. In transient transfection experiments, the choice of delivery vehicle is crucial for avoiding off-target effects such as immune gene perturbation that can potentially mask or change

the cellular response to the synthetic miRNA used. However, in certain clinical situations, such immune responses may be beneficial.

7. smiR-145 induced an immune response via RIG-I and was dependent on liposomal delivery as no immune gene expression changes were observed after delivery of smiR-145 directly into the cytosol using electroporation. Further, the liposome-induced immune response was different from the smiR-145 response and was not mediated by RIG-I.

Future studies

Scientific discoveries not only answer questions, but they also raise new questions to be answered. Here, I will mention some of the potential new studies that may be worth investigating further based on the findings in this thesis.

1. Besides miR-140-5p and miR-140-3p, the expression of several other miRNA also changed during in vitro culture of ACs and MSCs. The investigation of these miRNAs could reveal new and interesting biology. Such studies could also be extended by manipulating several miRNAs at the same time.

2. Performing ACI in an animal model with cells overexpressing miRNA mimics/inhibitors. In vitro studies are useful, but they do not always reflect what will occur in the body. In vivo studies would provide data that are more relevant for the clinical use of these cells.

3. Identify the mechanism responsible for post-transcriptional regulation of SOX9. This is an important goal of our future projects.

4. RALA was validated as a new target of miR-140-5p. Interestingly, RALA was shown to inhibit Activin signalling [280]. Furthermore, knockdown of Activin suppressed chondrogenesis, perhaps by regulating SOX9 [281,282]. This is worth investigating and may implicate RALA as a new and important regulator of cartilage development.

5. In paper III, we provide evidence showing that in certain situations, RIG-I depends on liposomal delivery of RNA for activation. This has not been described before and could be an interesting topic of future investigations of RIG-I biology.

6. Lipid-based transfection reagents mimic the properties of biological lipids to ensure fusion with cell membranes. Indeed biological lipids induce immune responses. The liposome-induced immune response reported in paper III induced many of the same genes that are upregulated when biological lipids induce an immune response in arthrosclerosis. It is possible that these two immune responses occur through the same mechanisms. We have been funded by The Research Council of Norway to investigate this possibility over next three years.

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I

II

III

Liposome delivery of microRNA-145 to mesenchymal stem cells leads to immunological off-target effects mediated by RIG-I

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Running title: Immune response against synthetic microRNAs

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Abstract

Synthetic microRNAs regulate gene expression when transfected into cells, and may be used in strategies for molecular therapy both in vitro and in vivo. Liposomal transfection reagents are frequently used as delivery vehicles in both settings. Here we report on the immunological off-target effects observed following liposome transfection of synthetic microRNA-145 into human mesenchymal stem cells and human articular chondrocytes. The immune response was independent on endosome delivery and toll-like receptors but was mediated by RIG-I. Upregulation of immune genes required liposomal delivery, as no immune response was observed after electroporation of smiR-145 directly in to the cytosol, suggesting a new role of RIG-I. Immune response was seen both with blunt ended and 2-nucleotide 3' overhang versions of synthetic miR-145, and occurred in the absence of a 5'ppp cap. Mutations in a centrally placed poly (UUUU) sequence reduced, but did not abolish the immune response. Interestingly, exposure to liposomes alone led to upregulation of several immune genes, including RIG-I mRNA. However, this process was not mediated by RIG-I. This insight is important for researchers to avoid unexpected results from gene transfer experiments in vitro and unwanted immune responses following the use of lipid-based transfection reagents in vivo.

Introduction

MicroRNAs (miRNAs) are a class of small endogenous RNA molecules that regulate gene expression. miRNAs are initially transcribed as long primary transcripts called pri-miRNAs. In the nucleus the pri-miRNAs are processed by the Drosha complex into precursor-miRNAs (pre-miRNAs). These are transported to the cytosol, where the Dicer complex cleaves the pre-miRNAs to yield mature and functional double stranded miRNAs approximately 21-22 nucleotides long. Usually, one of the strands is incorporated into the RNA-induced silencing complex (RISC) where it interacts with complementary sites in target mRNAs leading to either mRNA degradation or translational repression.[1,2] The end result will, in both cases, be reduced protein synthesis. However, miRNAs have also been shown to enhance protein synthesis.[3] To date the miRBase sequence database contains 2042 mature human microRNA sequences. Each miRNA can regulate many genes, and it has recently been estimated that >60% of all human genes are regulated by miRNAs.[4]

Transient transfection of synthetic microRNAs (smiRs) is often used in research protocols to investigate the function of miRNAs and genes. To yield specific and reliable results, these reagents must bind only to their designated RNA targets. However, liposome mediated transfection of siRNAs has been shown to induce off-target effects by upregulation of immune genes in different cell types.[5-8] This occurred through the sequence specific recognition of the siRNA by the endosomal RNA receptors toll-like receptor 3 (TLR3), TLR7 and TLR8.[7,9]

We recently performed a study describing the changes in miRNA expression as human articular chondrocytes dedifferentiate during *in vitro* cell culture.[10] Here, miR-145 was found to be strongly upregulated in dedifferentiated chondrocytes. To identify miR-145 targets and unravel molecular mechanisms of chondrocyte dedifferentiation we have subsequently performed liposome mediated transient transfection assays to overexpress miR-145 in human articular chondrocytes (hAC) and human bone marrow-derived mesenchymal stem cells (hBM-MSCs). The present study describes our observation that lipid-mediated transfection of smiR-145 induced an immunological off-target effect in hBM-MSCs and hACs. Surprisingly, the immune response was independent of endosomal delivery and endosomal TLRs, but was mediated by the cytosolic viral sensor retinoic acid inducible–gene 1 (RIG-I). The immune response was induced by smiR-145, but not by any of many other smiRs tested. smiR-145 sequences both with blunt ends and with 2 nucleotide overhangs induced immune response. The immune response was reduced following

mutations in a centrally placed UUUU sequence, but clearly depended also on other elements in the smiR-145 sequence. It required liposome delivery of smiR-145, as no immune gene changes were observed after delivery of smiR-145 directly into the cytosol using electroporation. This suggest a new role for RIG-I in certain situations, where RIG-I only recognize specific RNAs if it is delivered by liposomes. In fact, an immune response, albeit at lower level, was observed by exposing the cells to liposomes only. The liposome-induced immune response was not mediated by RIG-I.

Liposomal transfection reagents are frequently used as delivery vehicles in strategies for molecular therapy both in vitro and in vivo. Immunological off-target consequences of the use of liposomes and smiRs may confuse the interpretation of experiments, and potentially harm patients. Understanding the molecular mechanisms behind these effects and how to avoid them are therefore important if cell transfection is to be employed in clinical trials. These data may also open new lines of investigation into the understanding of RIG-I biology and perhaps also into the cellular immune responses to physiological lipids.

Results

Liposome mediated transfection of smiR-145 leads to upregulation of immune genes in mesenchymal stem cells

Our initial studies showed that liposome mediated transfection of smiR-145 led to greatly increased upregulation of a number of immune genes. To formally demonstrate this, and to identify all the changes in gene expression following either liposome-mediated transfection or electroporation of smiR-145 relative to a negative control RNA sequence (smiR-neg), we performed global mRNA array analysis using hBM-MSCs from three donors.

First, to evaluate our smiR transfection procedures, we established transfection of the positive control smiR-1, which is known to degrade *PTK9* mRNA. As shown in Supplementary Figure S1, transient transfection using liposomes (Lipofectamine 2000) and electroporation (Amaxa nucleofection) resulted in 80-85% and 55-60% knockdown compared to smiR-neg, respectively. Following liposome mediated transfection of smiR-145 into BM-MSCs, a total of 490 genes were upregulated and 209 genes were downregulated compared to the smiR-neg (Supplementary Table S2). The majority of the upregulated genes were associated with the immune system as shown by Gene Ontology (GO) analysis (Supplementary Table S3). Most significant, perhaps, was the strong upregulation of *INFB1*. Several of the upregulated genes are known to be involved in the regulation of *IFNB1*

expression, such as *TLR3*, *MYD88*, *DDX58* (also known as *RIG-1*), *IFIH1* (also known as melanoma associated gene 5, MDA5) and the transcription factor *IRF7* (interferon regulatory factor 7).[11] *IRF3*, the gene coding for one of the other important transcription factors involved in the activation of interferon β (IFNB) was not upregulated. However, RT-qPCR showed that *IRF3* was expressed at relatively high levels in these cells, while *IRF7* mRNA was expressed at low to moderate levels but increased following liposomal transfection of smiR-145, confirming the microarray data (Supplementary Figure S2). Liposomes alone (Mock) or liposomes with smiR-neg induced low level upregulation of *IRF7*. Downstream of the interaction of IFNB with its receptor, several STAT genes which were found to be upregulated in this experiment are known to induce upregulation of proinflammatory, apoptotic and antiviral genes.[12] Many of the upregulated genes encode products which act as chemoattractants and stimulants for immune cells such as monocytes, T cells and B cells (e.g. *CXCL10, CXCL11, CCL5, TNFSF13B, CCL8, CCL2, BST2),* others are known to have roles in virus resistance (e.g. *OAS1 OAS2* , *OAS3, OASL, RSAD2, MX1),* while yet others are pro-apoptotic *(*e.g.*TNFSF10, IFIT2).* A complete list of upregulated genes is presented in Supplementary Table S2.

The downregulated genes were a much more heterogeneous group, and were changed less dramatically than the upregulated genes. Most of the downregulated genes encode proteins with roles in cell metabolism. A surprising proportion was pseudogenes or non-protein coding genes. Some of the downregluated genes, such as *RPS23, RPL10A, RPL15, RPL23* and *RPLP0* are associated with ribosomes and translational elongation. Bioinformatic analysis using the miRWalk database predicted 14 % of the downregulated genes after liposomal transfection to be targets of smiR-145 (data not shown). Following transient transfection of smiR-145 using electroporation one gene was upregulated and nine were downregulated (Supplementary Table S4a). All the downregulated genes, except for the two ORFs, were also downregulated to the same levels in the liposomal smiR-145 transfected cells (Supplementary Table S4b) and have been shown to decrease after transfection with miR-145 mimics also in other studies.[13-15] miRwalk predicted seven of the nine genes (78%) to be miR-145 targets (data not shown). However, the number of miR targets suggested by downregulated genes following electroporation of miR-145 is likely to be falsely low in these cells, because the endogenous miR-145 level in hBM-MSCs was very high, and comparable to the highly abundant spliceosomal U6 snRNA (Supplementary Figure S3). This suggests that endogenous miR-145 had already downregulated its targets,

and that further overexpression by transient transfection of smiR-145 occurred in cells with few available miR-145 targets left.

Immune genes are upregulated by liposomes alone and further upregulated by smiR-145

To validate some of the upregulated genes from the microarray analysis by RT-qPCR analysis we chose *CXCL10, CCL5, OAS2* and *TLR3.* Assaying the same genes to determine the sequence specificity of this response we transfected other small RNAs: smiR-140, anti-miR-negative control (anti-neg), anti-145 and anti-140 in the same experiment. The results are shown in Figure 1a. For all the immune genes, liposome mediated smiR-145 transfection demonstrated greatly upregulated mRNA levels compared with untransfected controls. The levels of *CCL5* and *CXCL10* following transfection of all the RNA sequences by electroporation were similar to the untransfected controls. For liposome mediated transfection, none of the RNA sequences other than smiR-145 gave values above mock and smiR-neg transfection. However, for all the liposome mediated transfections, the levels of the immune genes were consistently higher than the levels obtained for electroporation, and those observed in untransfected controls. This shows that liposomes alone induced upregulation of immune genes. Using hACs we obtained the same results after transfection of mock, smiR-neg and smiR-145 (Figure 1b). The upregulation of CCL5 and CXCL10 following liposome mock transfection and transfection of smiR-neg and smiR-145 was confirmed at the protein level both in hBM-MSC and in hAC (Figure 2a, b). As part of another experiment, using cells from another donor, five more smiRs were used for liposome mediated transfection. Again, only smiR-145 increased *CCL5* and *CXCL10* levels above levels observed by mock transfection, further supporting specificity for smiR-145 for the immune gene upregulation (Supplementary Figure S4a, b). Supplementary Figures S4c and d show that there was a dose-response relationship for the liposome mediated immune response, and Supplementary Figures S4e, f and g show a similar dose-response relationship and kinetics for the smiR-145 induced immune response.

As shown in Supplementary Figure S1, we obtained better reduction of the miR-1 target gene *PTK9* for liposome mediated transfection than for electroporation. This could lead to the suspicion that the efficacy of the transfection was not sufficiently high to induce immune response when the smiRs were introduced by electroporation. From the liposome transfection dose-response experiments, using 25 nM of smiR-145, the immune genes were still greatly upregulated (Supplementary Figure S4e, f). In parallell experiments using 25 nM

of smiR-1 we saw approximately 60% knockdown of *PTK9,* which was exactly the same knockdown as that observed using electroporation (Supplementary Figure S5). This shows that the failure to obtain equal knock-down levels was not the reason why no immune response was observed when using electroporation of smiR-145.

To exclude the possibility that the liposome mediated immune response was restricted to Lipofectamine liposomes, we also tested siPORT NeoFX, which is also a cationic liposomal transfection reagent. siPORT NeoFX was not as efficient as Lipofectamine as determined by *PTK9* levels after transfection with smiR-1 (Supplementary Figure S6a). However, siPORT NeoFX reagent alone also increased *CXCL10* and *CCL5* mRNA levels, and a further increase was observed after transfection of smiR-145 (Supplementary Figure S6b,c).

smiRs are not taken up into endosomes, and the immune response is not induced by toll-like receptors

Endosomal toll-like receptors (TLRs) have been shown to be responsible for immune response against siRNAs after liposomal transfections.[7,9,16] Therefore, it has been thought that siRNAs transfected using liposomes are taken up by the endosomal pathway before being released into the cytosol. In the endosomes, TLR3 binds double-stranded RNA (dsRNA) and subsequently signal through the adaptor protein TRIF, while TLR7 and 8 bind single-stranded RNA (ssRNA) and signal through MYD88.[17,18] Our microarray analyses showed upregulation of both *TLR3* and *MYD88* mRNA following liposome mediated smiR-145 transfection, but *TLR7* and *TLR8* was not expressed in these cells (GEO database, accession number GSE40387). Suspecting that the immune response could be mediated through one of these pathways, we sought evidence for this by independently knocking down *TLR3* and *MYD88* by electroporation of siRNAs two days prior to liposomal smiR-145 transfection. *CXCL10* and *CCL5* mRNA expression was again chosen as readout. However, highly efficient knockdown of TLR3 and MYD88, did not decrease *CCL5* or *CXCL10* levels after liposomal smiR-145 transfection as would be expected if these were the pathways involved (Figure 3a, b). TLR3 is also expressed on the cell surface of certain cells. However, flow cytometry analysis showed that hBM-MSCs did not express TLR3 at the cell surface, and adding smiR-145 to the culture medium without liposomes did not lead to upregulation of either *CCL5, CXCL10, OAS2* or *TLR3* (data not shown). Also, two donors were treated with a MYD88 homodimerization inhibitory peptide before liposomal transfection of smiR-

145 and subjected to microarray and RT-qPCR analysis. No inhibitory effect on the immune response was observed compared to cells treated with a control peptide (data not shown). This strongly indicated that endosomal TLRs were not responsible for the immune response. To further explore this issue, we took advantage of the fact that endosomal TLRs depend on endosomal acidification for signaling.[19] Using chloroquine to inhibit endosomal acidification, we again observed highly upregulated levels of *CCL5* and *CXCL10* mRNA following liposomal smiR-145 transfection, and these levels were unaffected by chloroquine treatment (Figure 3c). Based on these results we wanted to see if the liposomes containing smiRs were taken up by the endosomal pathway at all. To this end we performed liposome transfection of a FAM-labeled smiR into hBM-MSCs. Using antibodies against the early endosome marker EEA1, the late endosome marker CD63 and the lysosome marker LAMP1, confocal microscopy was used to examine for co-localization following smiR transfection. Representative images are shown in Figure 4. Most liposomes seemed to stick together to form large aggregates, and these were not co-localized with EEA1, CD63 or LAMP1 staining vesicles. Co-localization with CD63 could be observed for very small smiR-FAM vesicles 45 minutes after transfection in one cell out of 19 observed, but this was never seen for EEA1 or LAMP1 (Figure 4). Combined, these experiments showed that the smiR-145 mediated immune response was independent of TLRs, and that the main uptake of liposome transfected smiRs did not involve the endosomal pathway. It is worth mentioning that none of the siRNAs used in this study induced an immune response after electroporation as determined by *CCL5* and *CXCL10* expression (data not shown).

smiR-145 induced immune response is mediated through RIG-I

As endosomal receptors could be excluded as being responsible for the smiR-145 induced immune response, our attention focused on cytosolic RNA receptors. mRNA levels for the cytosolic RNA binding receptors *PKR* (official gene symbol - *EIF2AK2*) and *RIG-I* (*DDX58*) were increased 3.9 and 18.1 fold, respectively, in our microarray analysis after liposomal smiR-145 transfection (Supplementary Table S2). However, delivery of smiR-145 directly into the cytosol by electroporation did not lead to upregulation of immune genes, suggesting that these cytosolic receptors were unlikely to be involved in the immune activation. Nevertheless, we still thought it could be possible that PKR or RIG-I, by an unknown mechanism, could be involved in the immune gene upregulation observed after liposomal smiR-145 transfection. Therefore, we first validated the increased *PKR* and *RIG-I*

expression in these donors. Both were found to be upregulated by liposomes alone (mock), and further upregulated by smiR-145 (Figure 5a, b). Then, we separately knocked down *PKR* and *RIG-I* before liposomal smiR-145 transfection. *PKR* knockdown did not affect the highly upregulated levels of *CCL5* or *CXCL10* resulting from the smiR-145 transfection (Figure 5c). RIG-I siRNA transfection resulted in 55-60% knockdown of *RIG-I* mRNA before smiR-145 transfection. After smiR-145 transfection *CCL5* and *CXCL10* mRNA levels were reduced by ~75-95% compared to scrambled control siRNA. Results from *RIG-I* knockdown were consistent in MSCs from 3 consecutive donors (Figure 5d). Protein levels of CCL5 and CXCL10 were measured in the supernatants from two of the donors and showed relative reductions after RIG knockdown similar to the mRNA levels (Figure 5e). Western blot analysis showed a band of approximately 101 kDa, which corresponds to the molecular weight (MW) of RIG-I, appearing 24 hours after smiR-145 transfection (Figure 5f). This band was not detected in cells treated with RIG-I siRNA. Another band with a MW around 90-95 kDa was also visible on the membrane. However, as this band did not change upon smiR-145 stimulation or by RIG-I siRNA treatment, this is likely to be a non-specific band. To rule out the possibility that the results from the RIG-I knockdown might be due to off-target effects of the siRNA used we tested another siRNA against *RIG-I* in two of the donors. The results were the same, although less pronounced (Supplementary Figure S7). These results strongly point to RIG-I as the mediator of the smiR-145 induced immune response.

smiR-145 induced immune response is mediated by RIG-I also in the HEK293 TN cell line

RIG I contain two caspase recruitment domains (CARDs) at its N-terminal which are responsible for signaling and an RNA binding domain at the C-terminal.[20] To further investigate the role of RIG-I in the smiR-145 induced immune response we overexpressed full length RIG-I (RIG-I), RIG-I without CARD domains (RIG-IΔCARDs), only the CARD domains (RIG-I CARDs) and control vector (EGFP) in the 293TN cell line followed by liposomal transfection of smiR-145. RT-qPCR analysis showed low *RIG-I* levels in this cell line unaffected by mock, smiR-145 and plasmid control transfection using primers which amplify a region within the *CARD1* and *CARD2* domains (Figure 6a, Supplementary Figure S8a). High levels of *RIG-I* were detected in cells transfected with full-length *RIG-I* and *RIG-I-CARDs*, but not after transfection with *RIG-IΔCARDs* (Figure 6a). Transfection of all

constructs resulted in cells expressing GFP and proteins of the correct size using antibodies specific for the C-terminus of RIG-I and GFP, respectively (Supplementary Figure S8b,c). Although low levels of *RIG-I* mRNA were detected in the 293TN cells, we did not detect endogenous RIG-I protein using western blot (Figure 6b, right lane). Overexpression of full-length RIG-I in the 293TN cells showed a band corresponding to the MW of RIG-I (Figure 6b, left lane). The lack of immune response in 293 TN cells, without prior overexpression of RIG-I, is consistent with previous findings where treatment of poly (I:C) did not induce immune response in 293T cells.[21] Overexpression of full-length RIG-I induced *CCL5* and *CXCL10* in the absence of smiR-145, but increased further by smiR-145 transfection (Figure 6c, d). The combination of full-length RIG-I and smiR-145 also induced CCL5 and CXCL10 protein secretion (Figure 6e, f). Transfection of *RIG-IΔCARDs* or *RIG-I CARD*s did not induce an immune response, either alone or combined with smiR-145 transfection, demonstrating that full-length RIG-I, containing both the RNA recognition and signaling domains, was required for upregulation of immune genes. Co-transfection of full-length *RIG-I* and *RIG-I* siRNA, followed by liposomal smiR-145 transfection, led to decreased mRNA and protein levels of CCL5 and CXCL10 compared to co-transfection of full-length *RIG-I* and scrambled control siRNA (Figure 6g, h). We also wanted to transfect a Cy3 labelled smiR-145 to evaluate possible co-localization with RIG-I/EGFP. However, the addition of Cy3 abolished the immune response completely in hBM-MSCs (data not shown). The labeling procedure leads to Cy3 attachment to both strands, but is not meant to affect the binding of smiR to its target mRNA. Presumably smiR-145 binding to RIG-I involves other parts of the smiR-145 molecule, and this interaction seems to be affected by Cy3 labeling.

Structure and sequence of smiR-145 of importance for immune gene upregulation

We have considered the possibility that technical aspects could explain some of our observations. However, in all these experiments five different batches of smiR-145 from Ambion were used, which makes it unlikely for "batch contamination" to be responsible for the immune response. smiR molecules produced by Ambion are double-stranded, 21-23 nt long with sequences identical to the sequence of the mature endogenous miRNA. The opposite strand is 100% complementary to the mature sequence. However, smiR molecules from Ambion have an unknown proprietary chemical modification, and have 2-nucleotide 3'overhang at each end which could possibly influence the immune response. In addition, the Ambion smiR molecules do not have a 5'triphosphate (5'ppp) which has been shown to

be important for RIG-I activation in several studies.[22-24] In contrast, smiRs from Qiagen do not contain any chemical modifications or overhangs (Supplementary Figure S9a). To determine the possible role played by the chemical modification and 3' overhangs, smiR-145 from Ambion and Qiagen were liposome transfected into MSCs in parallel. Figure 7a shows that smiR-145 from Qiagen (smiR-145-Q) induced *CCL5* and *CXCL10* to the same levels as smiR-145 from Ambion (smiR-145-A). The lack of immune response against smiR-1-Q confirms that the immune response was specific and not due to a general response to the Qiagen miRNA mimics. smiR-1-Q was also used in the positive control experiment for transfection efficiency (Supplementary Figure S9b).

Next we considered sequences within smiR-145 which might be important for the immune gene upregulation. The sequence 5'-GUCC-3' has been shown to be a potent immunostimulatory motif mediated by TLR7 (The United States Patent and Trademark Office (uspto.gov), Pat no: 8,076,068).[5] Both miR-145 and miR-642a contain this 4mer sequence (Supplementary Table S5). However, transfection of smiR-642a did not induce an immune response in hBM-MSCs (Figure 7b). Aligning the mature miR-145 sequence with sequences of dsRNA viruses (the RNA virus database) showed that several viruses contained the 5'UCCCAGG-3' sequence found within miR-145. miR-331 also contain this sequence (Supplementary Table S5). We then transfected smiR-331, but no immune response was observed (Figure 7b). Poly (U) sequences are known to activate RIG-I.[25] Both miR-145 and miR-140-5p contain a poly (UUUU) sequence in the same position in the mature sequence (Supplementary Table S5), but smiR-140 also did not lead to immune gene upregulation in our experiments (Figure 1a). In order to further explore the role of the poly (UUUU) sequence for smir-145 induction of immune genes we transfected two custom made sequences from Qiagen, one where the poly (UUUU) was changed into (UCCU) (smiR-145 2mut) and the other into poly (CCCC) (smiR-145 4mut). Figure 7c shows that smiR-145 2mut decreased *CCL5* and *CXCL10* by 50-60%, while smiR-145 4mut decreased the levels to 70-80% compared with the non-mutated smiR-145-Q. However, the immune response was not abolished by these mutations as the immune gene upregulation induced by smiR-145 4mut was still more than hundred times higher than mock and smiR-neg-Q transfections (Figure 7c). Still, the poly (UUUU) sequence is clearly important for smiR-145 binding to RIG-I, presumably combined with other parts of the smiR-145 sequence and possibly also the smiR-145 three-dimensional structure.

The liposome-induced immune response is not mediated by RIG-I

Several of the experiments described above showed that exposure to liposomes alone led to upregulation of immune genes. Our microarray analysis, on which the detection of upregulated immune genes, compared smiR-neg and smiR-145. Genes upregulated by liposomes alone and not further upregulated by smiR-145 would not be detected by the microarray analysis. We suspected that this might be the case for several important pro-inflammatory cytokines such as interleukin 6 (*IL6), IL8* and *IL1B,* shown by others to be upregulated following liposome mediated transfection of siRNA,[7] but not differently expressed in our microarray data. We therefore decided to analyse these genes by RT-qPCR in untransfected cells, cells exposed to liposomes alone (mock), liposomes plus smiR-neg and liposomes plus smiR-145. Figure 8a shows that these genes were upregulated in all the cells exposed to liposomes, but that they were not further upregulated by smiR-145. IL8 protein concentration in the culture medium followed the same pattern as the mRNA levels (Figure 8b). Further, we wanted to see if this immune response was also mediated by RIG-I. In three donors we knocked down RIG-I with siRNA before exposing the cells to liposomes. This did not lead to decreased levels of *CCL5, CXCL10, IL6, IL8* or *IL1B* after liposome exposure, demonstrating that the liposome induced immune response was not mediated by RIG-I (Figure 8c). Presumably the other genes shown to be upregulated by liposomes, i.e. *RIG-I* itself, *TLR3*, *OAS2*, *PKR* and *IRF7* are upregulated by the same mechanism.

Discussion

smiRs and and miRNA inhibitors hold great potential as therapeutic agents and are also important tools for identification of miRNA targets and their role in biological processes. A very common method for delivery of these molecules into cells is the use of liposome-based transfection reagents. Here we show that liposome-mediated transfection of smiR-145 induced upregulation of a substantial number of immune genes and the translation of their corresponding proteins in hBM-MSCs and hACs. The immune response was dependent on the liposomes, as no immune response was observed after electroporation of smiR-145. Surprisingly, the immune response was independent of uptake through endosomes and signaling through TLRs. Knockdown experiments demonstrated that the immune response was mediated by RIG-I. Interestingly, liposomes alone also induced a moderate immune gene up regulation, but this immune response was not mediated by RIG-I.

Exposure to exogenous RNA most commonly represents danger, and the cell has several strategies to evaluate the danger and deal with it. Many RNA viruses will be taken up into endosomes, where the viral dsRNAs are recognized by TLR3, and ssRNAs are recognized by TLR7 and TLR8. The TLRs will induce upregulation of immune genes to ensure the demise of the infecting virus. In the cytoplasm, short dsRNAs may be bound by PKR and OAS1-3.[26] PKR binding will induce an immune response or inhibit protein synthesis, while binding to members of the OAS family will predominantly lead to RNA degradation. Also in the cytoplasm, blunt-ended dsRNA may bind to RIG-I, MDA5 or LPG2. LPG2 lacks CARDs and thus cannot induce signalling, but may act as a negative regulator of RIG-I.[27] MDA5 binds long dsRNA (1-10 kb in length)[28] leading to upregulation of immune genes. RIG-I binds short dsRNA also leading to an antiviral response. mRNAs encoding the dsRNA endosomal receptor TLR3 and the adaptor protein MYD88 involved in TLR7 and TLR8 signaling were both upregulated in this study. However, TLRs were found not to be involved in the smiR-145 induced immune response based on the observations that fluorescent smiR did not co-localize with early or late endosomal markers, and the immune response was not abolished by inhibition of endosomal acidification or efficient knock-down of *TLR3* and *MYD88.* Also *TLR7* and *TLR8* were not expressed by these cells. Previous studies suggest that immunological off-target effects depend on cell type, delivery method and RNA sequence. While Sioud observed inhibition of liposomal siRNA-induced TNFα and IFNα production in adherent human peripheral blood mononuclear cells following chloroquine treatment, and a complete abrogation after electroporation of the same siRNA directly into the cytosol, suggesting an endosomal pathway, [7] Hornung et al. observed IFNα production following electroporation of siRNA into the cytosol of plasmacytoid dendritic cells.[5] As is the case for the innate immune response induced upon infection with certain RNA viruses, the pathway used by siRNAs and smiRs may vary between cell types.[11]

The fact that liposome mediated transfection was required, while electroporation of smiR-145 directly into the cytosol did not induce an immune response pointed away from RNA receptors in the cytosol. However, as TLRs could be excluded and the cytosolic receptors were actually upregulated after liposomal transfection of smiR-145, we decided to perform experiments to see if they could be involved. Of the cytosolic RNA receptors, PKR was excluded based on the observation that siRNA knockdown of *PKR* did not affect the immune response. MDA5 was considered unlikely because it is known to bind only long

dsRNAs. Upon siRNA inhibition of RIG-I, however, CCL5 and CXCL10 mRNA and protein was reduced compared to scrambled control siRNA. The identification of RIG-I as the receptor responsible for the smiR-induced immune response was further supported by experiments in the HEK293 TN cell line.

To explain exactly how liposome mediated transfection of smiR-145 led to miRINIR we need to reconcile the following observations: i) Liposomes alone induced an immune response that was different from that induced by smiR-145 transfection, but included upregulation of RIG-I mRNA. ii) RIG-I protein was detectable at 24 hours in cells exposed to liposomes plus smiR-145. The upregulation of RIG-I protein, and the miRINIR, was inhibited by *RIG-I* siRNA. iii) Electroporation of smiR-145 did not induce an immune response in these experiments. In this discussion, for physiological relevance, we speculate about similarities between the cellular responses to liposome/smiR-145 particles and RNA viruses.

First, the immune response to liposomes was RIG-I independent, but induced upregulation of *RIG-I* and a number of other immune genes. Others have also shown that liposomes can induce an innate immune response in exposed cells.[29] Recently, it was shown that fusion of virus envelopes or lipofectamine with cell membranes induced an immune response that was dependent on STING, a transmembrane protein in the endoplasmic reticulum, but the mechanism leading to the immune response was not revealed.[30] Similar to these studies we have not, determined exactly how liposomes induce an immune response. However, there are several possible lipid receptors in the cell membrane, some of which are known to induce upregulation of immune associated genes.[31,32] Also, many viruses enter cells through fusion with the plasma membrane. The actin cytoskeleton beneath the plasma membrane acts like a barrier for pathogens and is disassembled during virus entry.[33,34] Disassembly of the actin cytoskeleton has been shown to induce upregulation of immune genes.[35,36] This is the first level of immune response against enveloped viruses, and a similar mechanism may explain the liposome-mediated immune response reported here.

Second, the smiR-145 induced immune response was dependent on RIG-I. RIG-I is constitutively expressed in mouse embryonic fibroblasts,[37] and is frequently cited as being constitutively expressed also in human cells, albeit at low levels. In untransfected hBM-MSCs we observed very low level expression of *RIG-I* mRNA as determined by RT-qPCR. The RIG-I protein was only detectable 24 hours post-transfection. It has been shown that RIG-I is associated with the actin cytoskeleton beneath the plasma membrane.[38,39] If RIG-I

is sequestered to immediately below the plasma membrane, it may still be present in untransfected cells at functional concentrations although it was undetectable using our WB settings. Also, constitutively expressed RIG-I is normally found in a closed, non-responsive configuration.[40] It is conceivable that distortion and polymerization of the actin cytoskeleton, possibly brought about by fusion of liposomes with the plasma membrane or, in vivo, by fusion of the virus envelope with the plasma membrane, may activate RIG-I to enable binding to smiR-145 or virus dsRNA. Interestingly, RIG-I has been shown to directly interact with actin and also to regulate actin polymerization.[38]

Third, electroporation of smiR-145 did not bring about an immune response, despite small amounts of RIG-I protein presumably being present in the cells. One explanation for this may be the one described above, that the RIG-I is present only in the closed, non-responsive form and that electroporation does not affect the cells in a way that activates RIG-I. Another possibility is that RIG-I is sequestered immediately under the plasma membrane to such an extent that it is not available to bind smiR-145 introduced deeper within the cytoplasm by electroporation. This makes sense because it would be much more efficient for RIG-I to survey the actual pathways viruses utilize for cell entry instead of keeping the whole cytosol under surveillance. To the best of our knowledge, the dependency of liposome delivery for RIG-I activation has not been described before and open new lines of investigation into RIG-I biology. However, this phenomenon may differ between cell types and the RNA used for transfection.

In summary, then, our hypothesis is that liposomes fuse with the plasma membrane, which may trigger membrane-associated lipid receptors and/or distort the actin cytoskeleton which in turn upregulates immune genes. Further, we hypothesize that the smiR-145 induced immune response is separate from the immune response induced by liposomes alone, and is driven by the RIG-I RNA receptor. RIG-I may be sequestered immediately below the plasma membrane, in physical contact with actin as shown previously,[38,39] and smiR-145 activation of RIG-I sets off an immune response which in turn further upregulates RIG-I in a positive feedback loop.

The ligand for RIG-I in our study was smiR-145, and not any of another 11 smiRs and anti-miRs tested. This suggests that there is likely a restricted number of smiRs suitable as ligands in this system. Generally, short RNAs rich in GU or poly(U) motifs have been shown to be more likely to induce immune responses via RIG-I.[25] As such, it may not be surprising that the miR-145 sequence (5'-GUCCAGUUUUCCCAGGAAUCCCU-3') was

found to induce an immune response. Using other smiRs with sequence similarities that covered almost the entire smiR-145 sequence we were unable to identify short smiR-145 sequences which affected the RIG-I mediated immune response. However, by mutating the poly (UUUU) within the smiR-145 sequence we showed that this sequence is involved in the immune response. Still, a strong immune response was found even when the entire poly (UUUU) sequence was mutated, showing that also other parts of the smiR-145 sequence or three-dimensional structure are important for the immune gene upregulation.

RIG-I has been described to bind short blunt ended uncapped dsRNA, preferably with 5'ppp groups, but RIG-I has also been shown to bind dsRNA without 5'ppp and ssRNA containg 5'ppp.[21-24] The lack of cap is typical of viral sequences, and allows RIG-I to discriminate between self and viral dsRNAs.[41] Naturally occurring miRNAs processed by the Dicer complex have 2-nucleotide 3' overhangs. These overhangs impair the unwinding of the dsRNA substrate and inhibit immune gene induction through RIG-I, thus introducing another mechanism by which the cell can discriminate between self and nonself dsRNAs.[21] However, it has been shown that RIG-I are activated by RNA molecules containing overhangs.[42] In contrast to these previous findings, overhangs did not impact on the smiR-145 induced immune response in our experiments. This may be explained by use of different cells or by the inherent immunostimulatory properties within the different RNA sequences. It is possible that some RNA sequences are recognized by their ends, while others are recognized based on internal sequences or by secondary or tertiary structures that are not influenced by the end structures. Finally, a study by Witwer et al. demonstrated that a smiR mixture containing miR-145 reduced poly I:C induced IFN-β protein synthesis in macaque machrophages.[43] However, in contrast to the other smiRs in the mixture smiR-145 was not definitely shown to bind target sequences in the *IFNB* 3'UTR. Also, the possibility that liposome mediated transfection of smiR-145 alone might upregulate immune genes in these cells was not investigated – the immune response observed was entirely explained by the poly I:C treatment. Thus, if there are real discrepancies between the observations published in this study and those described here, they are likely to be due to technical differences and the use of different cells from different species.

The results described in this paper provide important information for researchers using different transfection techniques. Clearly an immune response can potentially mask or change the cellular response to the synthetic miRNA used, making it very difficult to interpret the actual role of the miRNA under investigation. Another concern is the unwanted

immune gene upregulation which may occur following liposome mediated transfection of smiRs in vivo. However, the induction and magnitude of the immune response may depend on cell type, transfection method and the reagents used.[44,45] As shown here, for hMSCs and hACs electroporation of smiRs is the in vitro transfection method of choice to avoid off-target immune responses. For researchers using other cells the recently established RNA immuno database provides an opportunity to analyse and compare the results of studies using RNA inhibition techniques,[46] and may be used as a guide to avoid or minimize immunological off-target effects in transfection experiments.

Materials and methods

Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Information about Taqman assays, smiRs and Anti-miRs, siRNAs, antibodies and singleplex arrays are listed in Supplementary Table S1.

Isolation and culture of cells

hBM-MSCs and hACs were isolated and cultured as previously described.[10,47] The hBM-MSCs fulfilled the criteria for definition of MSCs as proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy.[48] All donors provided written, informed consent. The study was approved by the Regional Committee for Medical Research Ethics, Southern Norway, Section A. From the day of isolation until the first passage cells were expanded in DMEM/F12 (Gibco, Paisley, UK) supplemented with 20% FBS (Cambrex, East Rutherford, NJ), 100 units/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B. The AC culture medium also contained 50µg/ml ascorbic acid. Culture medium was changed every 3-4 days. After the first passage amphotericin B was removed and 10% FBS, instead of 20%, was used. At 50-60% confluence cells were detached with trypsin-EDTA and seeded into new culture flasks. For hBM-MSCs, 12 different donors were used for consecutive experiments. All experiments were performed on cells between passage 3 and 6, in log phase of growth. For AC the experiments were performed on cells from two donors at passage 3 and 4. HEK293 TN cells (System Biosciences, CA) were cultured in DMEM/F12 - high glucose (Gibco) supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin.

Plasmids

RIG-I constructs (all fused with GFP) were kindly provided by Carolyn B. Coyne (University of Pittsburgh, Department of Microbiology and Molecular Genetics). The EGFP-RIG-I plasmid codes for full-length RIG-I (aa1-925) with GFP fused at its N terminus.[39] The EGFP-RIG-IΔCARDS construct (aa201-925) does not contain the CARD1 and CARD2 domains necessary for CARD-CARD interaction with the downstream adaptor protein MAVS and subsequent signalling. However, it contains the HELICASE domain responsible

for RNA recognition and helicase activity. The EGFP-RIG-I CARDS construct (aa1-225) contains CARD1 and CARD2, but not the HELICASE domain. The control construct EGFP only contains EGFP.

Transfection

Lipofectamine 2000 was used for liposomal transfection according to the reverse transfection protocol from the manufacturer (Invitrogen, Carlsbad, CA). Briefly, for each reaction 500.000 cells were transfected using 50 nM smiRs (double-stranded RNA) or anti-mirs (single-stranded RNA) (Ambion, Austin, TX) and 8 ul Lipofectamine 2000, and were seeded in a total volume of 2.5 ml culture medium without antibiotics. Other concentrations of smiRs and Lipofectamine 2000 were used for dose-response experiments as stated in the figures. siPORT NeoFX transfection reagent (Ambion) was used in one experiment. HEK293 cells were transfected according to the forward transfection protocol (Invitrogen) using 300.000 cells, 8 µl Lipofectamine 2000, 500 ng plasmid DNA, 50 nM smiRs with or without 150 nM siRNA in a total volume of 2.5 ml.

The Amaxa Nucleofection system was used according to the protocols from the manufacturer (Lonza, Walkersville, MD) (http://www.lonzabio.com/technology.html). For both hBM-MSCs and hACs the Human Chondrocyte Nucleofector Kit and the U-23 program was used as these conditions were shown in pilot experiments to give the best transfection efficiency for both cell types. Briefly, 1 x10^6 cells were used for each nucleofection reaction using 3 µM smiRs or anti-miRs in a total volume of 100 µl nucleofector solution. Directly after transfection the cells were seeded in culture medium without antibiotics containing 20% FBS. Transfection of siRNAs was performed with the Amaxa nucleofection system at a final concentration of 1.5 µM for each siRNA two days prior to liposomal transfection.

For inhibition of endosomal acidification, chloroquine (InvivoGen, San Diego, CA) was added to the culture medium at 0, 0.1, 1, 10, 50, 100 or 500 µM 2 hours prior to transfection and was also included in the culture medium during transfection.

For all transfections the culture medium was changed into regular culture medium without antibiotics 20-24 hours after transfection, and samples were harvested for analysis 2 days post transfection unless otherwise stated.

Inhibition of MYD88

MYD88 signaling was blocked by incubating hBM-MSCs with a MYD88 inhibitory peptide and a control peptide following protocols in the MyD88 Homodimerization Inhibitory Peptide Set (Imgenex, San Diego, CA).

mRNA and miRNA quantification – RT-qPCR

Total RNA for mRNA analysis was isolated using the RNAqueous Micro Kit following protocols from the manufacturer (Ambion). Following DNase treatment (Ambion), RNA was quantified by spectrophotometry (Nanodrop, Wilmington, DE). From each sample 200 ng total RNA was reverse transcribed into cDNA (total volume of 20µl) by using the High Capacity cDNA Reverse Transcription kit following protocols from the manufacturer (Applied Biosystems, Abingdon, U.K.). RT-qPCR was performed using Taqman® Gene Expression assays and Taqman Universal PCR master mix (Applied Biosystems) with the 7300 Real-Time RT PCR system (Applied Biosystems) following protocols from the manufacturer. All samples were run in technical triplicates. Each replicate contained 1.0 µl cDNA in a total volume of 25 µl. The thermocycling parameters were 95ºC for 10 minutes followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. *GAPDH* was used as endogenous control as its expression was not affected by transfection.

Total RNA enriched with small RNA molecules (including miRNA) was isolated using the miRNeasy Mini Kit (Qiagen, by, land) following protocols from the manufacturer. Following DNase treatment, the total RNA was quantified by spectrophotometry. Reverse transcription was performed using 10 ng total RNA (total volume of 15µl) and primers included in the Taqman® microRNA assays (Table 1) (Applied Biosystems). For RT-qPCR Taqman® MicroRNA Expression assays (Table 1) the Taqman Universal PCR master mix was used, and all samples were run in technical triplicates. Each replicate contained 1.33 µl cDNA in a total volume of 20 µl. The same thermocycling parameters as described above were used. *U6* was used as endogenous control.

Microarray analysis

Microarray analysis was performed at the Norwegian Microarray Consortium. Nanodrop (Wilmington, DE) and Bioanalyzer (Agilent, Santa Clara, CA) was used to check the concentration and integrity of the samples, respectively. For each sample 500 ng total RNA was amplified and labeled with biotin using Illumina® TotalPrep RNA Amplification Kit (Ambion). The biotin labeled cRNA (750 ng) was hybridized onto Illumina's HumanHT-12 v4 Expression BeadChip and subsequently stained with streptavidin-Cy3. The chips were then scanned using Illumina® BeadArray™ Reader. Results were imported into Illumina GenomeStudio v. 2010.2 Gene Expression v. 1.7.0. for data extraction and initial quality control. Further quality control was performed with the microarray analysis software J-Express[49] and included log(2) transformation, quantile normalization, box plot analysis, correspondence analysis and hierarchical clustering. Differential expression between samples was calculated using Rank product (RP).[50] A threshold of 2-fold and q-value <0.05 (adjusted p-value) was used to generate lists with differently expressed genes. Gene Ontology (GO) overrepresentation analysis (Bonferroni correction, $p < 0.05$) was performed on the gene lists from the RP-analysis. The guidelines from the "Minimal Information About a Microarray Experiment" (MIAME guidelines) were followed and the microarray data are available in the GEO database with accession number GSE40387.

Bioinformatic analysis

The miRWalk prediction database was used to predict miR-145 binding sites in the 3'UTR of the downregulated genes from the microarray analysis. Comparative analysis was performed by 6 prediction programs (miRanda, PICTAR5, miRDB, PITA, miRWalk and TargetScan). A positive prediction in 4 out of the 6 programs was chosen as criteria for being counted as a true prediction.

Cytokine measurements

The concentration of secreted CCL5 and CXCL10 in the culture medium was determined using singleplex assays from Biorad (Biorad, Hercules, CA) and the Luminex system (Luminex, Austin, TX) following protocols from the manufacturer.

Western blotting

Cell lysates corresponding to 200.000 cells were loaded onto an 8% polyacrylamide gel and transferred to PVDF membranes. After 1h blocking in 5% milk the membrane was incubated overnight with antibodies against RIG-I followed by washing and incubation with horse radish peroxidase conjugated secondary antibodies. The Precision Plus Protein All Blue Standards was used to determine molecular weights (Biorad). Pierce ECL western blotting substrate solution (Thermo Scientific, Runcorn, UK) was used for development and pictures were captured using the Carestream Image Station 4000 R Pro and the Carestream MI software (Carestream, Rochester, NY). The membrane was washed and reprobed with antibodies against β-actin (Abcam, Cambridge, UK) which was used as loading control.

Confocal microscopy

To determine if liposomes containing smiRs were delivered to the endosomal pathway, hBM-MSCs were transfected with a FAM-labeled smiR. At indicated time points cells were fixed with 4% paraformaldehyde for 15 min and then permeabilised with 0.1% Triton X-100 for 30 min. After washing, cells were incubated with primary antibodies against EEA1, CD63 and LAMP-1 for 1h at room temperature, washed again, incubated with secondary antibody for 30 min at room temperature, washed and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Primary antibodies were kindly provided by Anne Simonsen (University of Oslo, Department of Medical Biochemsistry, Institute of Basic Medical Science). Confocal images were acquired on an Olympus FluoView 1000 inverted microscope equipped with a PlanApo 60/1.10 oil objective (Olympus, Center Valley, PA). Images were analysed with ImageJ 1.46i (National Institutes of Health, Bethesda, MD)

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EGFP-RIG-IΔCARDS, EGFP-RIG-I CARDS and EGFP control plasmids. We would also like to acknowledge Esben Østrup for technical assistance with the confocal imaging and Julie K. Lindstad for technical assistance with the Luminex experiments.

Supplementary Material

Supplementary Figure S1. Validation of transfection efficiency.

Supplementary Figure S2. *IRF3* and *IRF 7* expression.

Supplementary Figure S3. Endogenous miR-145 expression

Supplementary Figure S4. Liposomal transfection of smiRs, dose-response and time kinetics.

Supplementary Figure S5. Transfection of smiR-1 using different concentrations

Supplementary Figure S6. Transfection using siPORT liposome transfection reagent

Supplementary Figure S7. RIG-I knockdown using siRNAs from Qiagen

Supplementary Figure S8 RIG-I construct expression in 293 TN cells

Supplementary Figure S9. Comparison of smiR-145 sequences from Ambion and Qiagen and *PTK9* knockdown using smiR-1 from Qiagen.

Supplementary Table S1. Summary of reagents used

Supplementary Table S2. Spreadsheet (separate excel file) containing microarray data comparison between lipofectamine transfection of smiR-neg and smiR-145 in hBM-MSCs

Supplementary Table S3. Gene Ontology analysis

Supplementary Table S4a. Genes differently expressed after electroporation of smiR-145

Supplementary Table S4b. Downregulated genes common for electroporation and lipofectamine

Supplementary Table S5. Sequence alignment of miR-145 with miR-642a, miR-331 and miR-140

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Figure legends

Figure 1 Validation of selected genes upregulated in microarray analysis by RT-qPCR. (a) Expression of *CCL5, TLR3, CXCL10* and *OAS2* was determined in untransfected cells (untr) and after liposome transfection of mock and different smiRs and anti-miRs in hBM-MSCs as indicated in the figure. *CCL5* and *CXCL10* were also determined following transfection by electroporation. Circle, triangle and rhombus symbols represent mean values of technical triplicates from three different donors. The mean ± SEM of the biological triplicates are shown by vertical lines. (b) Expression of *CCL5, TLR3, CXCL10* and *OAS2* in untransfected hAC and after liposome transfection of mock and indicated smiRs. *CCL5* and *CXCL10* were also determined following transfection by electroporation. Circle and rhombus symbols represent mean values of technical triplicates from two different donors. The mean ± SEM of the biological duplicates are shown by vertical lines. ND = not detected.

Figure 2 Cytokine secretion. (a) Concentration of CCL5 and CXCL10 proteins secreted into the culture medium by hBM-MSCs. Technical duplicates were used for each donor. Data are shown as mean ± SEM of the biological triplicates. (b) Concentration of CCL5 and CXCL10 proteins secreted by hACs. Technical duplicates were used for each donor. Data are shown as mean ± SEM of biological duplicates. ND = not detected.

Figure 3 Knockdown of TLR signaling and inhibition of endosomal acidification. Cells were treated with scrambled siRNA, TLR3 siRNA and MYD88 siRNA by electroporation (a) two days before liposomal transfection of smiR-145. Before the smiR-145 transfection the *TLR3* and *MYD88* knockdown were measured using RT-qPCR (white bars) using cells from one hBM-MSC donor and shown as mean ± SD of technical triplicates. *CCL5* and *CXCL10* mRNA levels were measured one day after smiR-145 transfection (grey bars). (b) shows knockdown of TLR3 and MYD88 at the protein level. The effect of different doses of chloroquine on the expression of *CCL5* (c) and *CXCL10*, (d) following smiR-145 liposomal transfection. Circle and rhombus symbols represent mean values of technical triplicates from two donors. Also 100 μM and 500 μM chloroquine was used, but all cells died at these concentrations and the results are therefore not included. CQ = chloroquine. ND = not detected.

Figure 4 Confocal microscopy of smiR delivery. Cells (hBM-MSCs from one donor) were transfected using a FAM-labeled smiR. At indicated time points cells were stained with antibodies against EEA1, CD63 and LAMP1. Green = FAM-labeled smiR, red = EEA1, CD63 or LAMP1, blue = DAPI. Scalebar = 10 µm

Figure 5 Validation and knockdown of *PKR* and *RIG-I* expression. Expression of *PKR* **(**a) and *RIG-I* (b) after transfection of mock and indicated smiRs. hBM-MSCs were treated with scrambled, PKR (c) and RIG-I (d) siRNA by electroporation two days before liposomal transfection of smiR-145. Before the smiR-145 transfection the *PKR* and *RIG-I* knockdown was measured using RT-qPCR. *CCL5* and *CXCL10* mRNA levels were measured one day after smiR-145 transfection. In (c) the results from one donor are shown as mean ± SD using technical triplicates. In (d) the circle, triangle and rhombus symbols represent mean values of technical triplicates from three different donors. The mean ± SEM of the biological triplicates are shown by vertical lines. In (e) the concentration of CCL5 and CXCL10 proteins secreted into the culture medium is shown as the mean ± SEM of the results from two of the donors, showing that protein levels were also efficiently reduced after smiR.145 transfection when the cells had been pre-treated with RIG-I siRNA.. Technical duplicates were used for each donor. In (f) cells were treated with scrambled and RIG-I siRNA by electroporation two days before smiR-145 liposomal transfection. Western blotting using antibodies against RIG-I were performed 6 and 24 hours after transfection (one hBM-MSC donor). Predicted molecular weight of RIG-I is 101 kDa. * = non specific bands.

Figure 6 Overexpression and knockdown of RIG-I in 293TN cells. (a) *RIG-I* mRNA levels were determined after liposomal transfection of mock, smiR-145, control construct (EGFP) and different RIG-I constructs as indicated in the figure. (b) A representative Western blotting image using antibodies against RIG-I in untransfected (right lane) and RIG-I transfected 293 TN cells (full length RIG-I + GFP, predicted molecular weight approximately 130 kDa). (c) *CCL5* and (d) *CXCL10* mRNA were measured after liposome transfection of indicated smiRs and plasmids. Protein levels of CCL5 (e) and CXCL10 (f) were measured after liposome transfection of indicated smiRs and plasmids. (g) mRNA and (h) protein levels after liposome co-transfection of RIG-I siRNA and full-length RIG-I plasmids relative to co-transfection of scrambled control siRNA and full-length RIG-I plasmid. mRNA levels (in a, c, d and g) are shown as mean ± SD from technical triplicates.

Protein levels (in e, f and h) are shown as mean ± SD from technical duplicates. ND = not detected.

Figure 7 Induction of miRINIR using smiR-145 and smiR-145 mutants from Qiagen. *CCL5* and *CXCL10* were measured after transfection of smiR molecules from Ambion (A) and Qiagen (Q) (a). smiR-642a and smiR-331 from did not lead to immune response (smiRs from Ambion) (b). Mutating the poly (UUUU) of smiR-145 reduced the immune response (c). Results in (a) and (b) are from two different donors and shown as mean ± SD from technical triplicates. In (c) circle, triangle and rhombus symbols represent mean values of technical triplicates from three donors. The mean ± SEM of the biological triplicates are shown by vertical lines. Numbers in parentheses indicate % expression relative to smiR-145-Q. ND = not detected.

Figure 8 Immune response to liposomes following mock transfection and transfection of smiR-neg and smiR-145 in hBM-MSCs. Quantification of interleukin gene expression of *IL6, IL8* and *IL1B* using RT-qPCR (a) and IL8 protein synthesis using the Luminex system (b). In (a) circle, triangle and rhombus symbols represent mean values of technical triplicates from three different donors. The mean ± SEM of the biological triplicates are shown by vertical lines. In (b) technical duplicates were used for each donor. Data are shown as mean ± SEM of the biological triplicates. In (c) cells were treated with scrambled and RIG-I siRNA by electroporation two days before liposomal mock transfection. Before the mock transfection *RIG-I* knockdown was measured using RT-qPCR. *CCL5*, *CXCL10, IL6, IL8* and*, IL1B* mRNA levels were measured one day after mock transfection. Circle, triangle and rhombus symbols represent mean values of technical triplicates from three different donors. The mean ± SEM of the biological triplicates are shown by vertical lines.

Figure 1

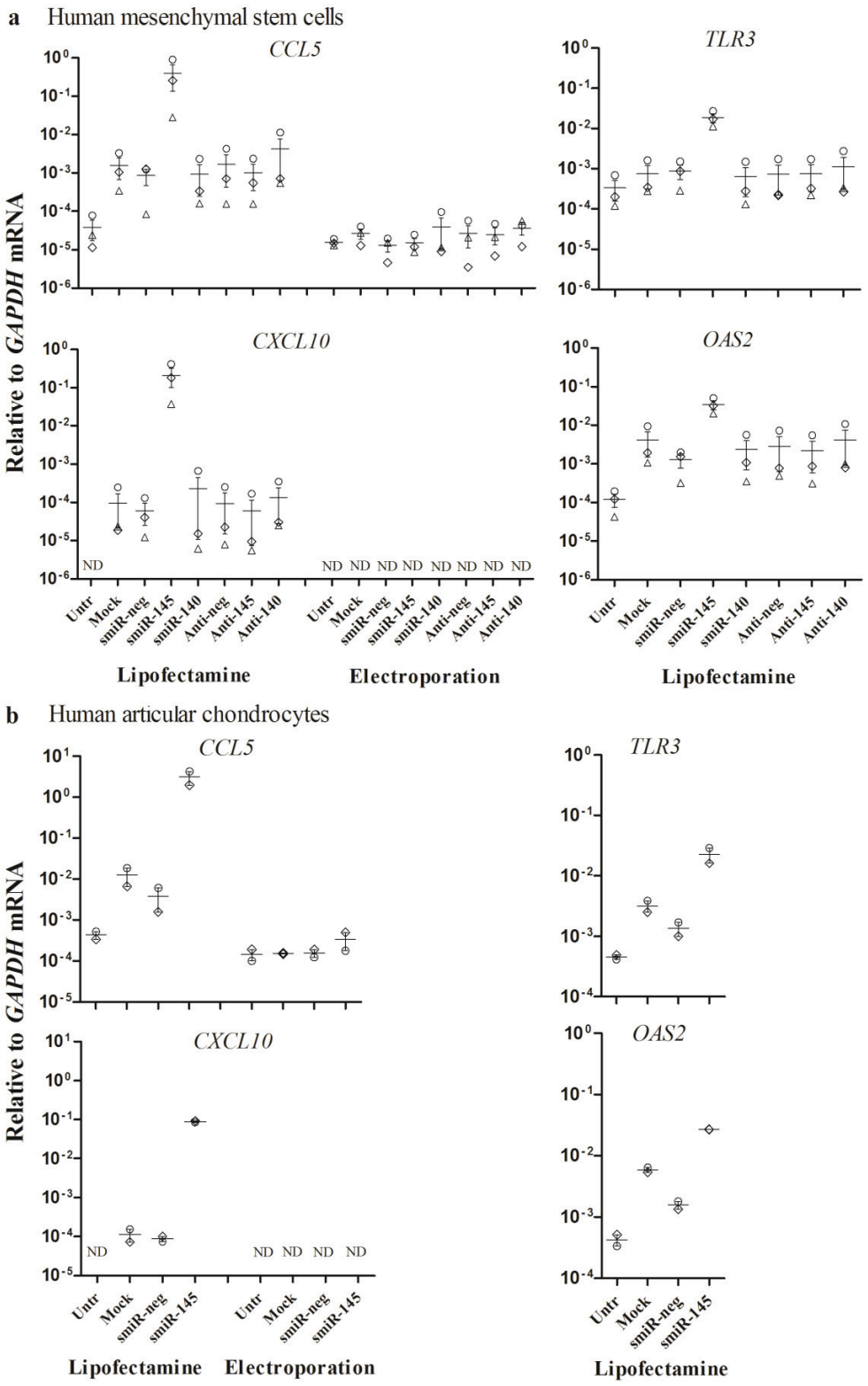

a
Human mesenchymal stem cells
CCL5
TLR3
CXCL10
OAS2
Relative to GAPDH mRNA
ND
Untr
Mock
smiR-neg
smiR-145
smiR-140
Anti-neg
Anti-145
Anti-140
Lipofectamine
Electroporation
Lipofectamine
b
Human articular chondrocytes
CCL5
TLR3
CXCL10
OAS2
Relative to GAPDH mRNA
Lipofectamine
Electroporation
Lipofectamine

Figure 2

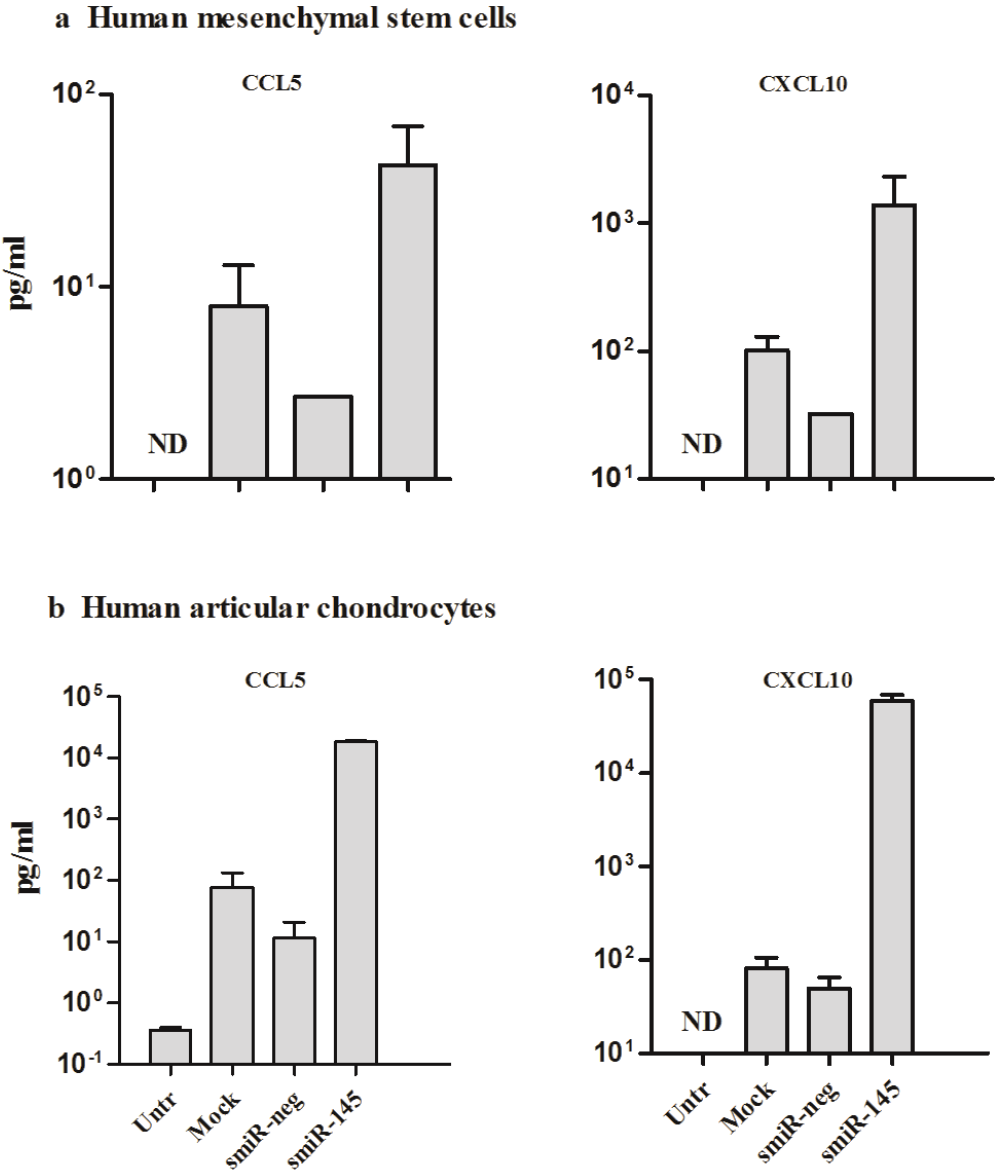

a Human mesenchymal stem cells
CCL5
CXCL10
pg/ml
ND
ND
b Human articular chondrocytes
CCL5
CXCL10
pg/ml
ND
Untr
Mock
smiR-neg
smiR-145

Figure 3

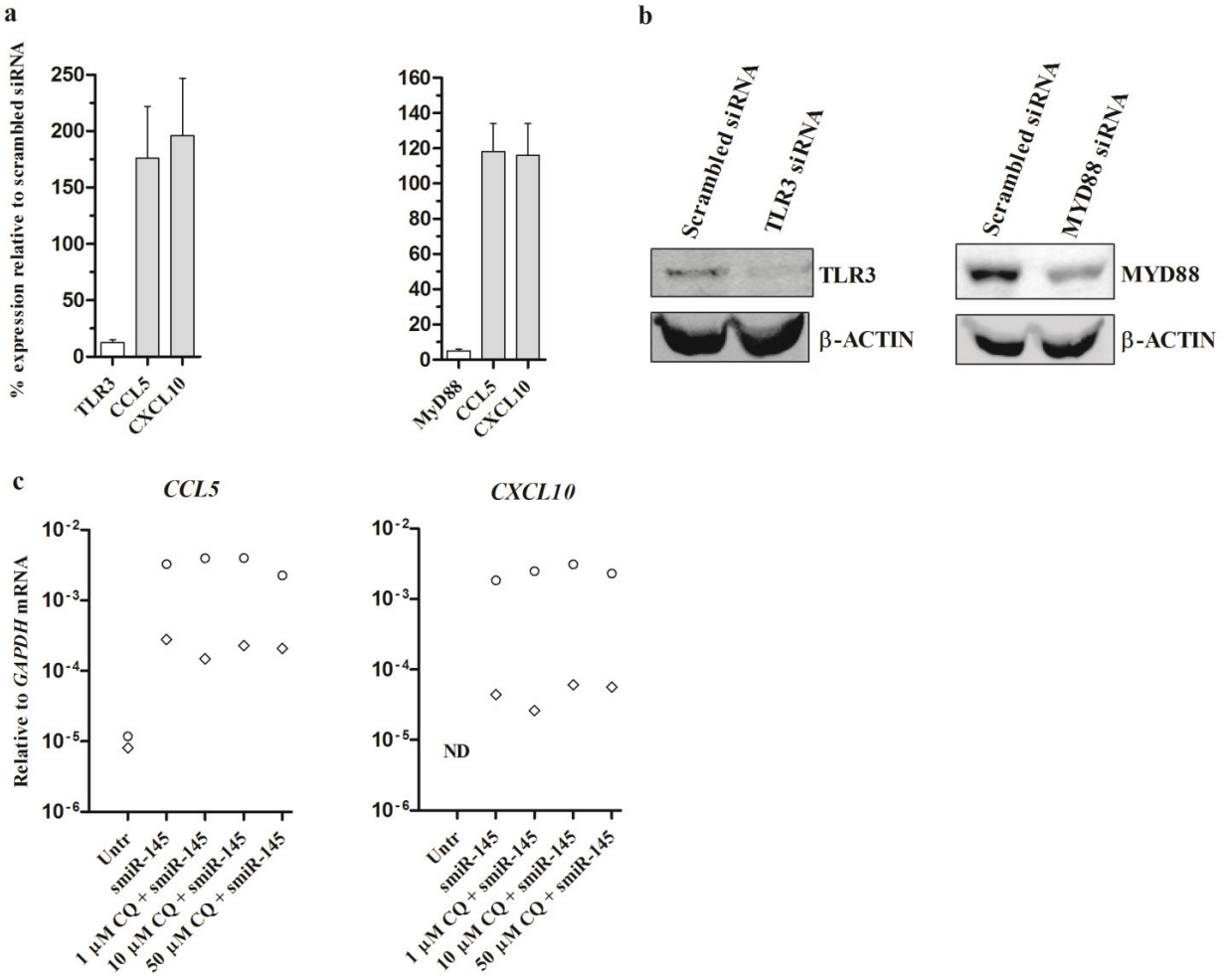

a
% expression relative to scrambled siRNA
250
200
150
100
50
0
TLR3
CCL5
CXCL10
160
140
120
100
80
60
40
20
0
MyD88
CCL5
CXCL10
b
Scrambled siRNA
TLR3 siRNA
TLR3
β-ACTIN
Scrambled siRNA
MYD88 siRNA
MYD88
β-ACTIN
c
CCL5
CXCL10
Relative to GAPDH mRNA
10-2
10-3
10-4
10-5
10-6
ND
Untr
smiR-145
1 μM CQ + smiR-145
10 μM CQ + smiR-145
50 μM CQ + smiR-145

Figure 4

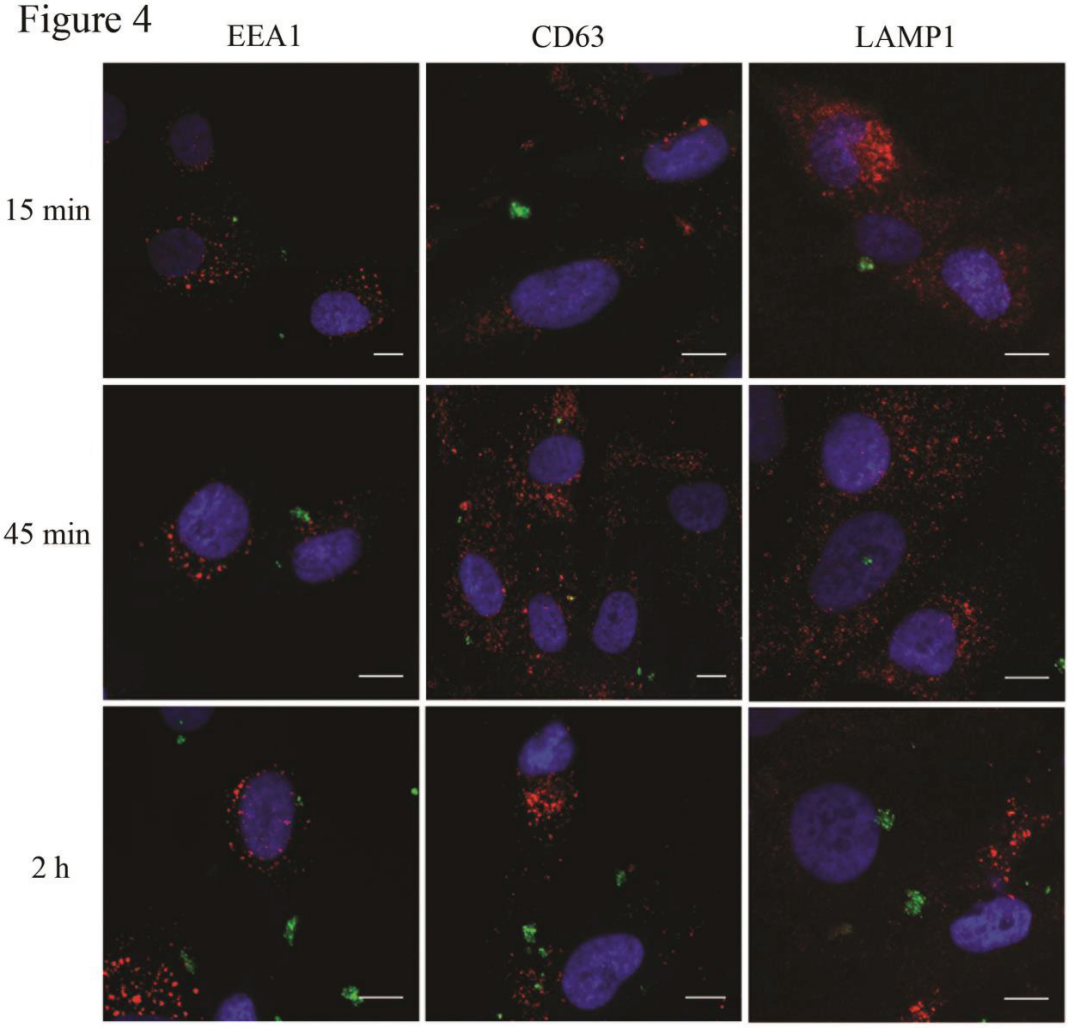


Figure 5

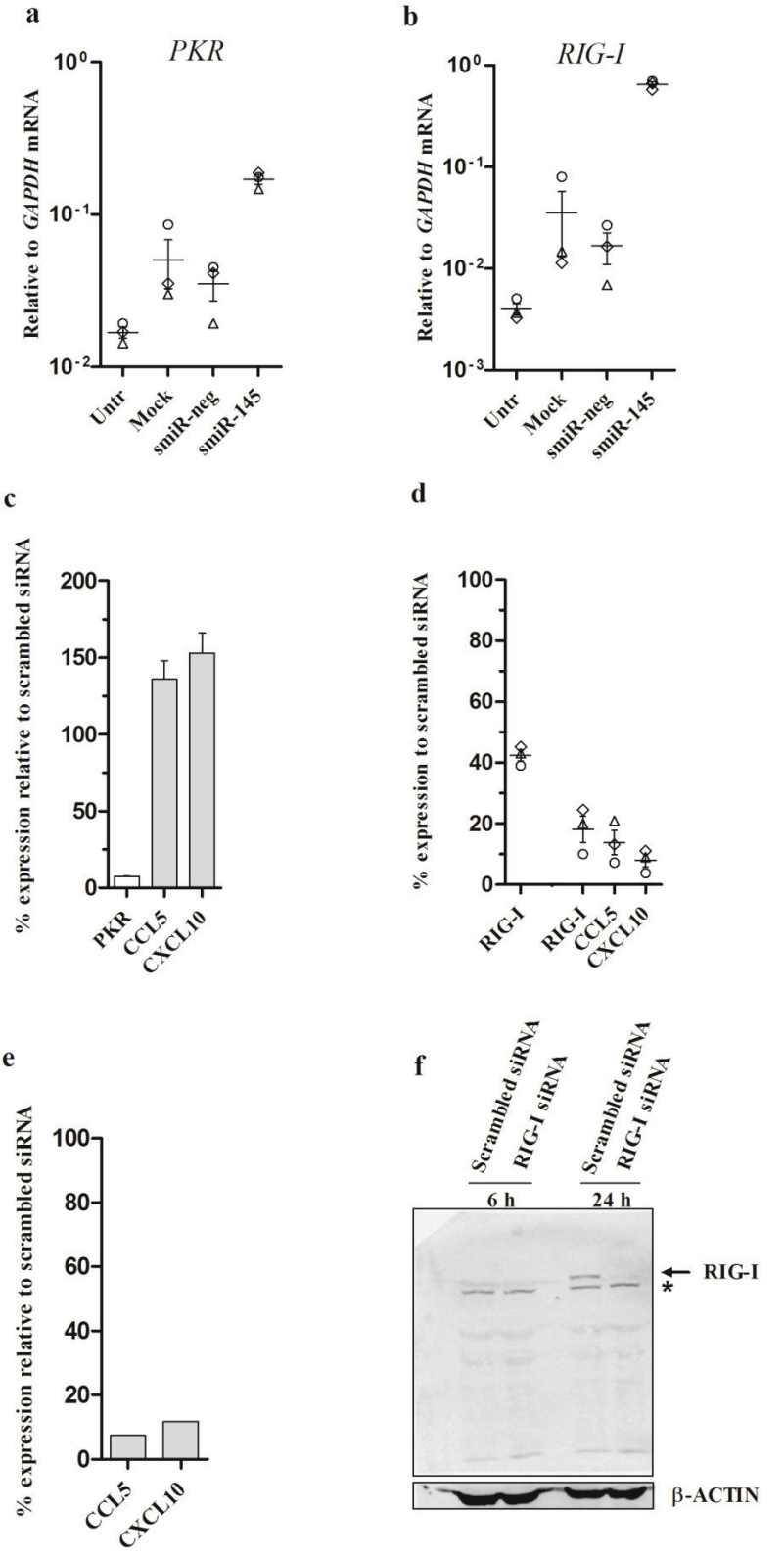

a
PKR
Relative to GAPDH mRNA
10^0
10^-1
10^-2
Untr
Mock
smiR-neg
smiR-145
b
RIG-I
Relative to GAPDH mRNA
10^0
10^-1
10^-2
10^-3
Untr
Mock
smiR-neg
smiR-145
c
% expression relative to scrambled siRNA
200
150
100
50
0
PKR
CCL5
CXCL10
d
% expression to scrambled siRNA
100
80
60
40
20
0
RIG-I
RIG-I
CCL5
CXCL10
e
% expression relative to scrambled siRNA
100
80
60
40
20
0
CCL5
CXCL10
f
Scrambled siRNA
RIG-I siRNA
Scrambled siRNA
RIG-I siRNA
6 h
24 h
RIG-I
*
β-ACTIN

Figure 6

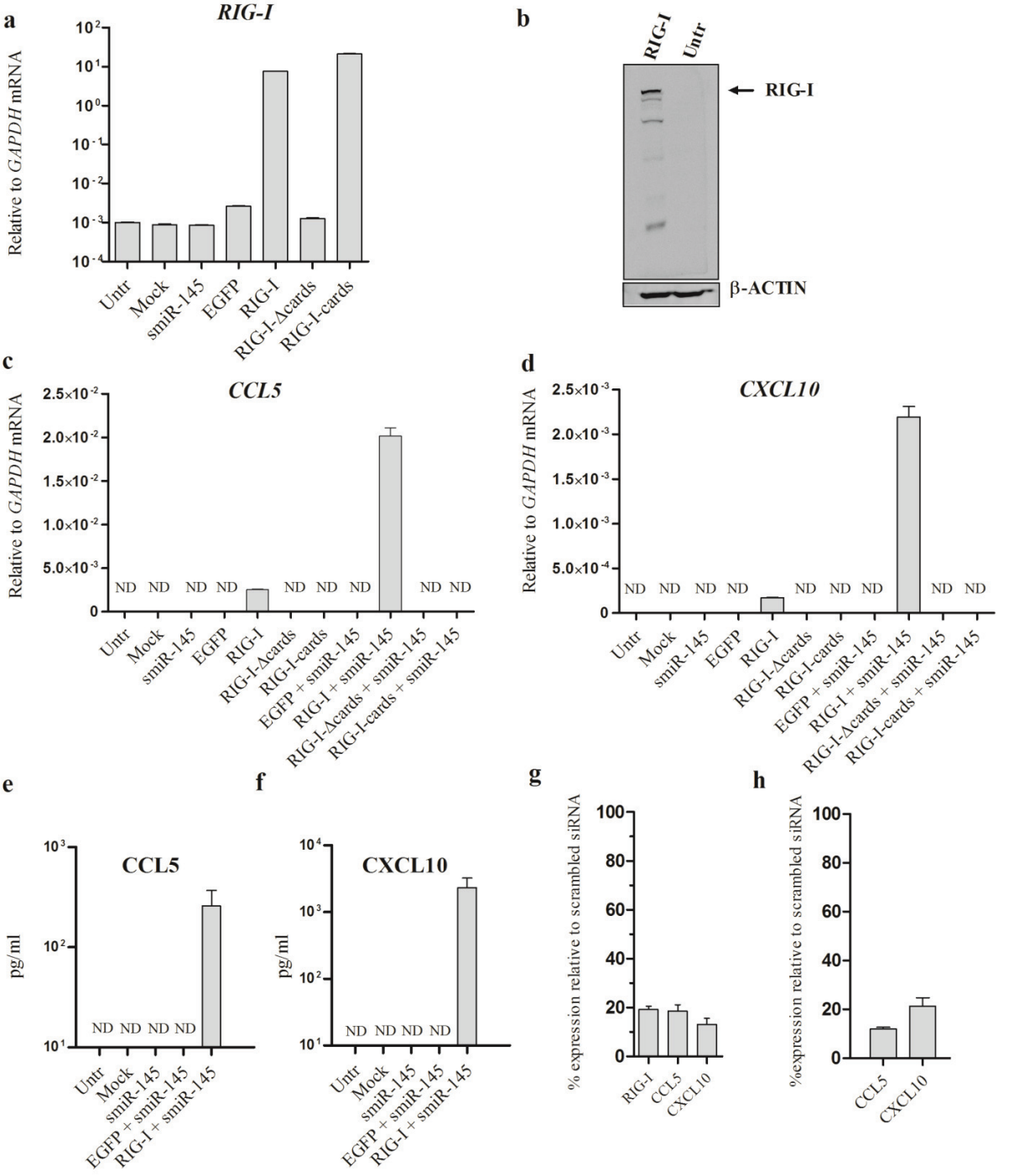

a
RIG-I
Relative to GAPDH mRNA
Untr
Mock
smiR-145
EGFP
RIG-I
RIG-I-Δcards
RIG-I-cards
b
RIG-I
Untr
RIG-I
β-ACTIN
c
CCL5
Relative to GAPDH mRNA
ND
EGFP + smiR-145
RIG-I + smiR-145
RIG-I-Δcards + smiR-145
RIG-I-cards + smiR-145
d
CXCL10
Relative to GAPDH mRNA
e
CCL5
pg/ml
f
CXCL10
pg/ml
g
% expression relative to scrambled siRNA
RIG-I
CCL5
CXCL10
h
%expression relative to scrambled siRNA
CCL5
CXCL10

Figure 7

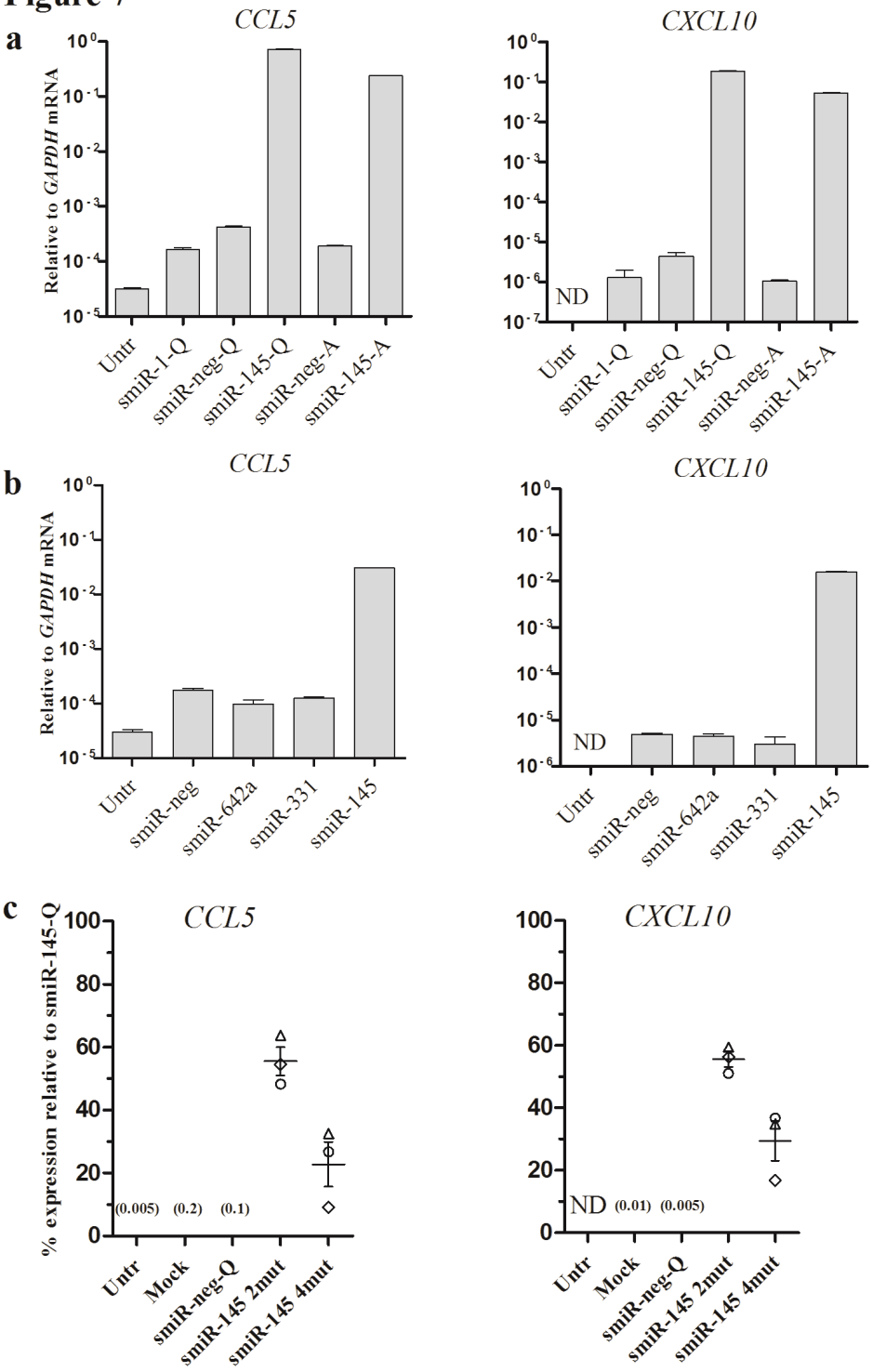
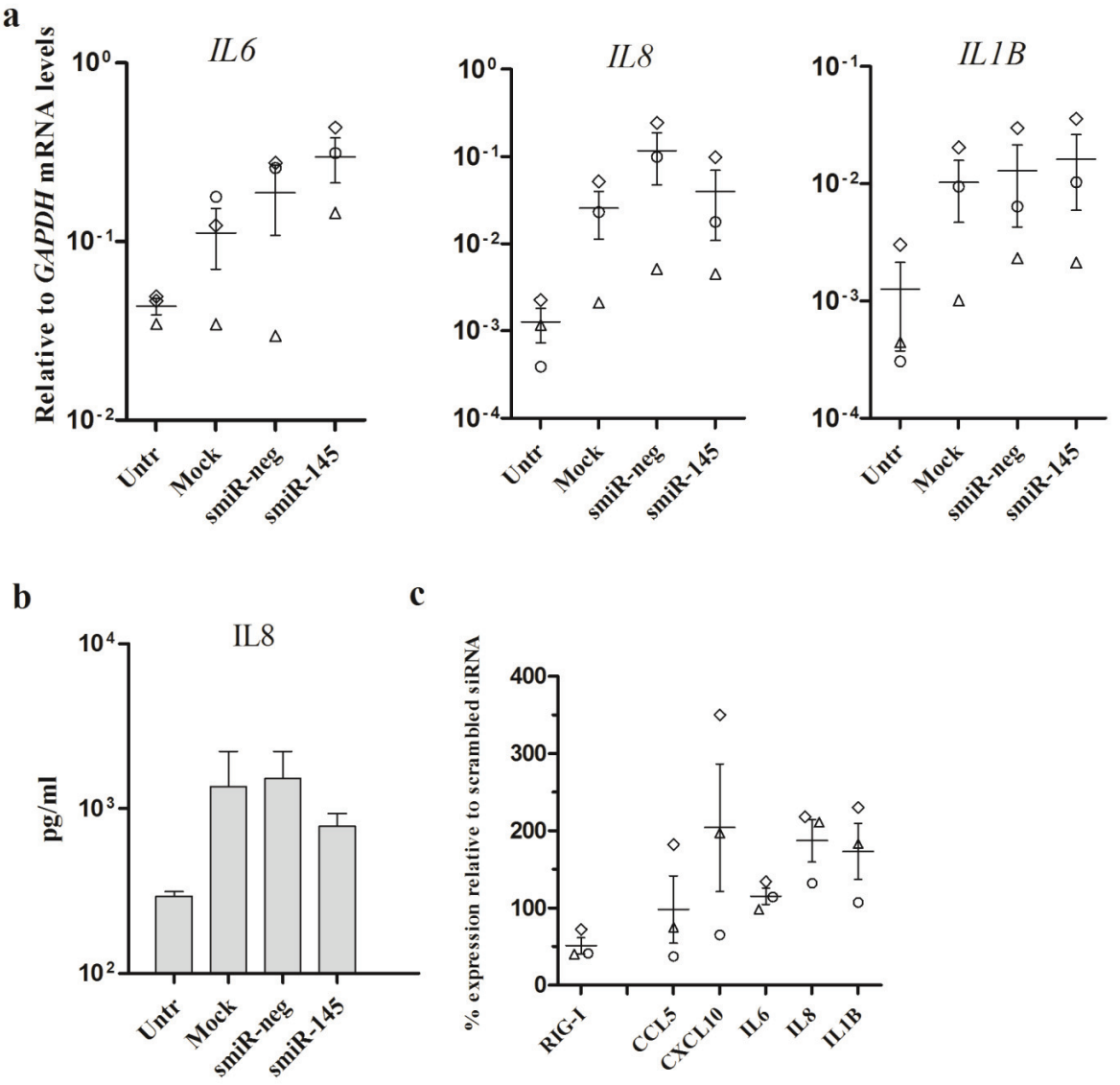
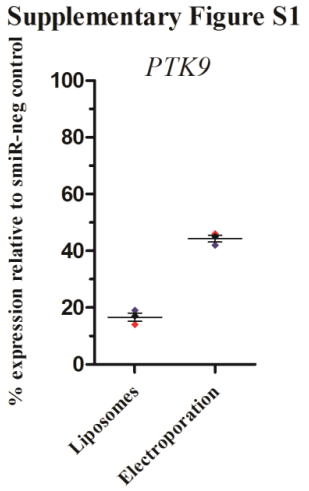

a
CCL5
CXCL10
Relative to GAPDH mRNA
ND
Untr
smiR-1-Q
smiR-neg-Q
smiR-145-Q
smiR-neg-A
smiR-145-A
b
CCL5
CXCL10
Relative to GAPDH mRNA
ND
Untr
smiR-neg
smiR-642a
smiR-331
smiR-145
c
CCL5
CXCL10
% expression relative to smiR-145-Q
(0.005)
(0.2)
(0.1)
ND
(0.01)
(0.005)
Untr
Mock
smiR-neg-Q
smiR-145 2mut
smiR-145 4mut

Figure 8

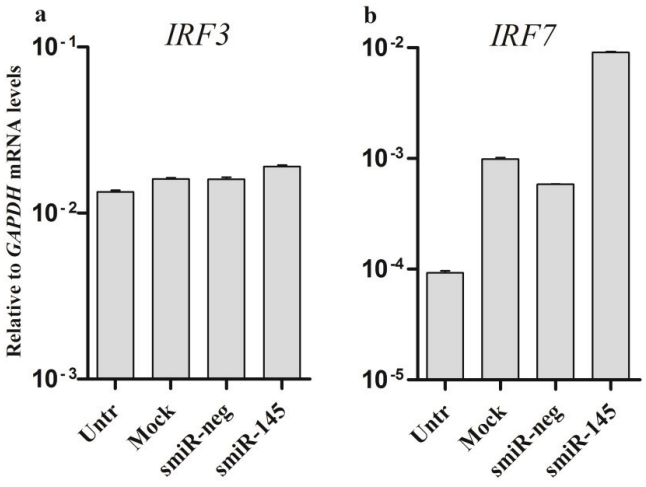

a
IL6
IL8
IL1B
Relative to GAPDH mRNA levels
Untr
Mock
smiR-neg
smiR-145
b
IL8
pg/ml
c
% expression relative to scrambled siRNA
RIG-1
CCL5
CXCL10
IL6
IL8
IL1B

Supplementary material



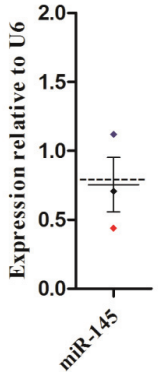
Supplementary Figure S1. Effective delivery and activity of smiR control. smiR-1 was used as a positive control as recommended by the manufacturer. smiR-1 should lead to degradation of *PTK9* mRNA. *PTK9* levels were measured by RT-qPCR after transfection of smiR-neg control and smiR-1 using liposomes or electroporation. Black, blue and red symbols represent mean values from technical triplicates from three different donors. The mean ± SEM of the biological triplicates are shown by vertical lines.

Supplementary Figure S2



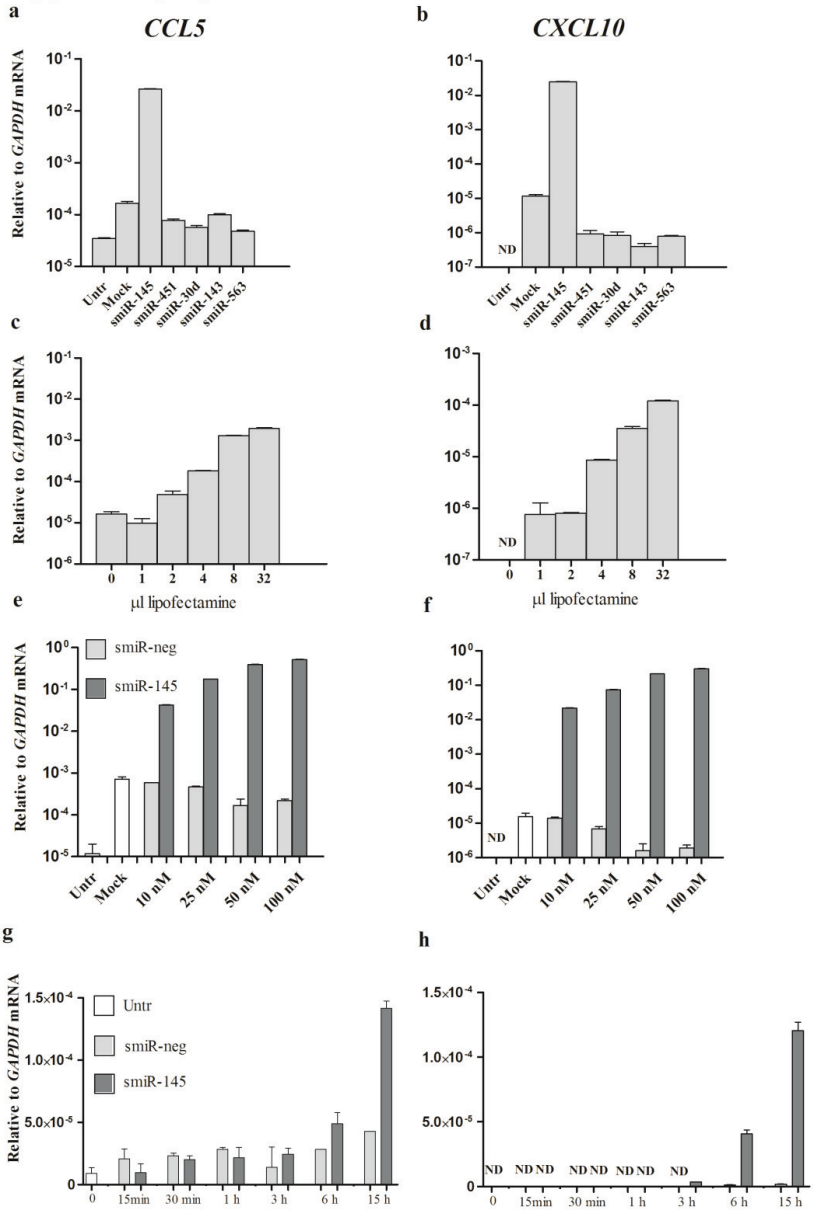
Supplementary Figure S2. Expression of (A) *IRF3* and (B) *IRF7* mRNA was determined in untransfected (untr) cells and after transfection of mock, smiR-neg and smiR-145 (hBM-MSC donor 1). Results are shown as mean ± SD from technical triplicates.

Supplementary Figure S3



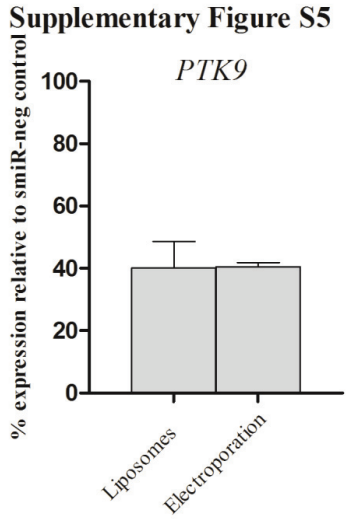
Supplementary Figure S3. Endogenous expression of miR-145 relative to U6 in untransfected cells determined using RT-qPCR. Black, blue and red symbols represent mean values of technical triplicates from three different donors (hBM-MSC donor 1, 2 and 3). The mean ± SEM of the biological triplicates are shown by vertical lines**.**

Supplementary Figure S4



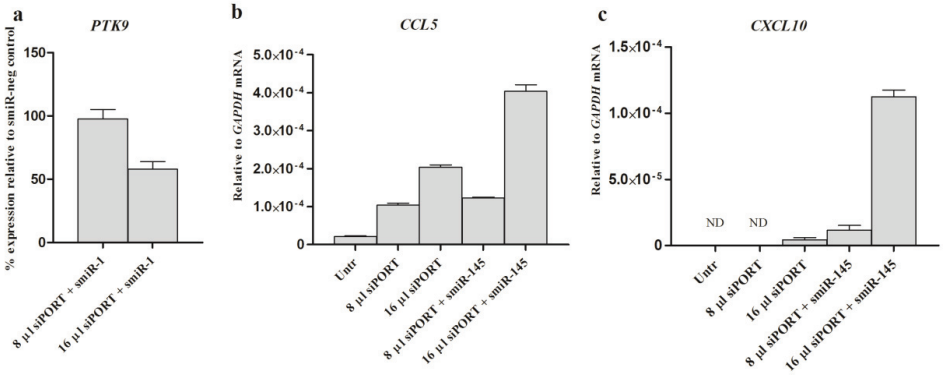
Supplementary Figure S4 Liposomal transfection of smiRs, dose-response and time kinetics. Expression of *CCL5* (a), (c), (e), and (g) and *CXCL10* (b), (d), (f), and (h) after liposomal

transfection of different smiRs (a, b, hBM-MSC donor 4), in response to different doses of lipofectamine (mock) (c, d, hBM-MSC donor 5, passage 3), in response to different concentrations of smiR-145 (e, f, hBM-MSC donor 5, passage 4) and in the course of a time kinetics of gene expression experiment (g, h, hBM-MSC donor 6). All results are shown as mean ± SD from technical triplicates using cells from one donor. ND = not detected.



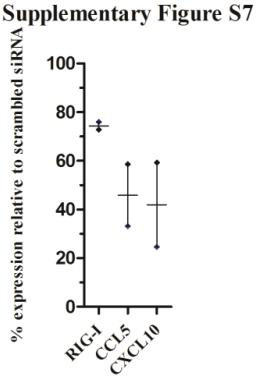
Supplementary Figure S5. Transfection of smiR-1 using liposomes (Lipofectamine) at 25 nM results in the same knockdown of *PTK9* mRNA as electroporation of smiR-145 (3 µM). Results are shown as mean ± SD from technical triplicates of one donor (hBM-MSC donor 5).

Supplementary Figure S6



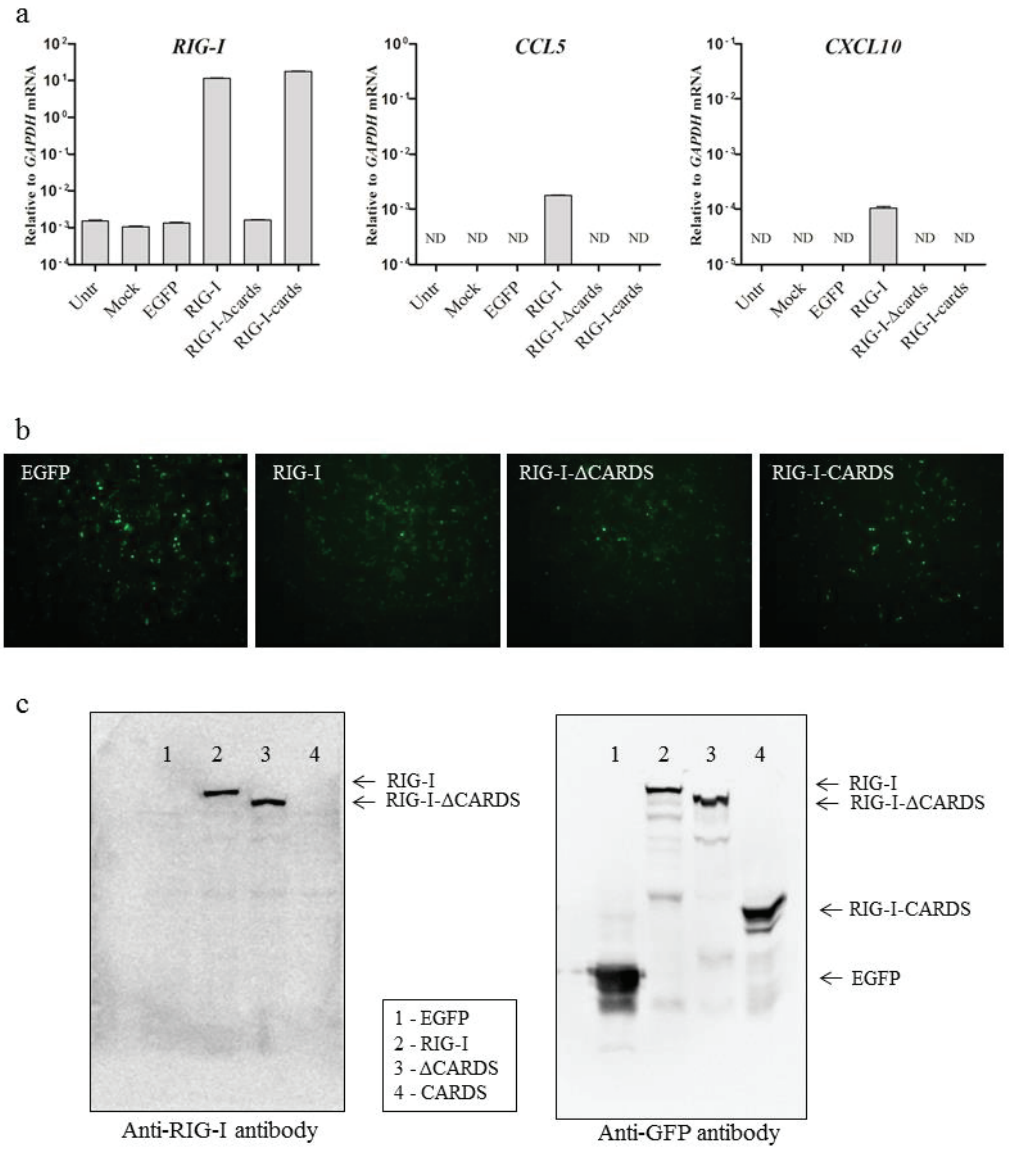
Supplementary Figure S6. Transfection using siPORT liposome transfection reagent. (a) Knockdown of *PTK9* after transfection of smiR-1. Expression of *CCL5* (b) and *CXCL10* (c) mRNA in untransfected cells and after transfection of mock (8 and 16 µl siPORT) and smiR-145 as indicated in the figure. Results are shown as mean ± SD from technical triplicates of one donor (hBM-MSC donor 5). N.D = not detected.

Supplementary Figure S7



Supplementary Figure S7. RIG-I knockdown using siRNAs from Qiagen. Cells were treated with scrambled and RIG-I siRNA by electroporation two days before liposomal transfection of smiR-145. Before the smiR-145 transfection *RIG-I* knockdown was measured using RT-qPCR. *CCL5* and *CXCL10* mRNA levels were measured one day after smiR-145 transfection. Black and blue symbols represent mean values of technical triplicates from two different donors (hBM-MSC donor 9 and 10). The mean ± SEM of the biological duplicates are shown by vertical lines.

Supplementary Figure S8



Supplementary Figure S8 Transfection of RIG-I constructs in 293TN cells. (a) shows mRNA expression of *RIG-I, CCL5* and *CXCL10* after transfection of the different construct as indicated on the X-axis. Results are shown as mean ± SD from technical triplicates. All constructs resulted in GFP expression (b) and all the expressed proteins had the expected size as checked by western blotting using anti-RIG-I and anti-GFP antibodies (c).

Supplementary Figure S9

a

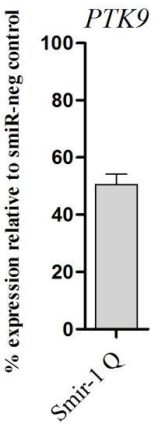
smiR-145 – Ambion

Mature microRNA sequence 5′-GUCCAGUUUUCCCAGGAAUCCCU-3′
Complementary sequence 3′-TTCAGGUCAAAAGGGUCCUUAGG-5′

smiR-145 Qiagen

Mature microRNA sequence 5′-GUCCAGUUUUCCCAGGAAUCCCU-3′
Complementary sequence 3′-CAGGUCAAAAGGGUCCUUAGGGA-5′

b



Supplementary Figure S9 Comparison of smiR-145 sequences from Ambion and Qiagen and PTK9 knockdown using smiR-1 from Qiagen. The 2 nucleotide 3'-overhang of the mature strand of Ambions smiR molecules always consist of the 2 last nucleotides from the mature endogenous miRNA sequence, while the overhang at the complementary strand is always a TT (a). Knockdown of *PTK9* after smiR-1-Q transfection was used as a positive control (b). Results are shown as mean ± SD from technical triplicates of one donor (hBM-MSC donor 11).

Supplementary Table S1. Summary of some of the reagents used

Taqman Assays			
Gene symbol	**Gene name**	**Company**	**Cat no.**
CXCL10	Chemokine (C-X-C motif) ligand 10	Applied Biosystems	Hs00171042_m1
CCL5	Chemokine (C-C motif) ligand 5	Applied Biosystems	Hs00174575_m1
TLR3	Toll-like receptor 3	Applied Biosystems	Hs01551078_m1
MYD88	Myeloid differentiation primary response gene 88	Applied Biosystems	Hs00182082_m1
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	Applied Biosystems	Hs00213443_m1
DDX58 (RIG-I)	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	Applied Biosystems	Hs00204833_m1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Applied Biosystems	Hs99999905_m1
TWF1 (PTK9)	Actin-binding protein, homolog 1	Applied Biosystems	Hs00702289_s1
EIF2AK2 (PKR)	Eukaryotic translation initiation factor 2-alpha kinase 2	Applied Biosystems	Hs00169345_m1
MicroRNA/small RNA assays			
Interim/gene symbol	**RNA name**	**Company**	**Cat no.**
Mir145	miR-145	Applied Biosystems	001141
RNU6-1 (U6)	RNA, U6 small nuclear 1	Applied Biosystems	001973
smiR and anti-miR molecules			
Name	**Sequence 5'-3'**	**Company**	**Cat no.**
Pre-negative control no 1	N/A	Ambion	AM17110
FAM-labeled Pre-negative control no 1	N/A	Ambion	AM17121

Pre-miR-145	guccaguuuucccaggaaucccu	Ambion	PM11480
Pre-miR-140-5p	cagugguuuuacccuaugguag	Ambion	PM10205
Pre-miR-30d	uguaaacaucccgacuggaag	Ambion	PM10756
Pre-miR-143	ggugcagugcugcaucucuggu	Ambion	PM10883
Pre-miR-451	aaaccguuaccauuacugaguu	Ambion	PM10286
Pre-miR-563	agguugacauacguuuccc	Ambion	PM11418
Pre.miR-642a	gucccucuccaaaugugucuug	Ambion	PM11477
Pre-miR-331	cuagguaugguccagggaucc	Ambion	PM11179
Pre-miR-1 (positive control)	uggaauguaaagaaguauguau	Ambion	AM17150
Anti-negative control no 1	N/A	Ambion	AM17010
Anti-miR-145	N/A	Ambion	AM11480
Anti-miR-140-5p	N/A	Ambion	AM10205
miScript-145	guccaguuuucccaggaaucccu	Qiagen	MSY0000437
miScript-1	uggaauguaaagaaguauguau	Qiagen	MSY0000416
Silencer select Pre-design and validated siRNA			
Name		**Company**	**Cat no.**
Silencer negative control no 2		Ambion	AM4637
TLR3		Ambion	s235
MYD88		Ambion	s9138
PKR		Ambion	s11185
RIG-I		Ambion	s24143
Allstars negative control		Qiagen	1027280
RIG-I		Qiagen	SI03019646
Antibodies			
Primary antibodies	**Protein name**	**Company**	**Cat.no**
Mouse anti-EEA1	Early endosome antigen 1		
Mouse anti-CD63	CD63 molecule	Developmental Studies	H5C6

		Hybridoma Bank	
Mouse anti-LAMP1	Lysosomal-associated membrane protein 1	Developmental Studies Hybridoma Bank	H4A3
Mouse anti-GFP	Green fluorescent protein	Clontech	632381
Goat anti-RIG-I (C-15)	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	Santa Cruz	sc-48929
Mouse anti-TLR3	Toll-like receptor 3	Abcam	Ab13915
Rabbit anti-MYD88	Myeloid differentiation primary response gene 88	Abcam	Ab1333739
Mouse anti-ACTB	Actin, beta	Abcam	Ab8226
Secondary antibodies		**Company**	**Cat no.**
Donkey anti-goat (HRP)		Jackson ImmunoResearch	705-035-147
Goat anti-Rabbit (HRP)		Vector labs	PI-1000
Horse anti-Mouse (HRP)		Vector labs	PI-2000
Donkey anti-mouse (Cy3)		Jackson ImmunoResearch	715-165-151
Singleplex assays		**Company**	**Cat no.**
Rantes (CCL5)		Biorad	171B5025M
IP-10 (CXCL10)		Biorad	171B5020M
IL-8		Biorad	171B5008M

N/A – not available

Supplementary Table S2. See separate file at the end

Supplementary Table S3

Gene Ontology analysis of genes differently expressed after Lipofectamine transfection of smiR-145

Upregulated

GO term
Regulation of viral protein levels in host cell
Intracellular transport of viral proteins in host cell
Antigen processing and presentation of peptide antigen via MHC class I
Antigen processing and presentation of endogenous peptide antigen
Lymphocyte chemotaxis
Base conversion or substitution editing
Antigen processing and presentation of endogenous peptide antigen via MHC class I
Negative regulation of viral reproduction
Antigen processing and presentation of peptide antigen
Regulation of defense response to virus by host
MHC class I receptor activity
MHC class I protein complex
MHC protein complex
Antigen processing and presentation
Positive regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains
Regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains
MHC protein binding
Negative regulation of interferon type I production
Cytokine and chemokine mediated signaling pathway
Regulation of interferon type I production
Interferon type I production
Defense response to virus
Response to virus
Response to cytokine stimulus
Positive regulation of inflammatory response
Chemokine activity

Downregulated

GO term
Translational elongation
Viral genome expression
Viral transcription
Translational termination
Viral infectious cycle
Endocrine pancreas development
Viral reproductive process
Structural constituent of ribosome
Ribosome
Endocrine system development
Taxis
Chemotaxis
Translation
Actin binding
Viral reproduction
Cell proliferation
Structural molecule activity
Oxidoreductase activity
Cytosol
System development
Protein binding
Multicellular organismal development
Cellular metabolic process
Cellular protein metabolic process
Cellular macromolecule metabolic process
Cytoplasmic part

Regulation of defense response to virus
Chemokine receptor binding
Regulation of smooth muscle cell proliferation
JAK-STAT cascade

Cellular process
Cytoplasm
Developmental process
Catalytic activity

Supplementary Table S4a

Genes differently expressed after electroporation of smiR-145

Upregulated

Gene symbol	Fold Change	q-value
ACTG2	2,24	0,003

Downregulated

Gene symbol	Fold change	q-value
TMEM9B	-3,13	0
FSCN1	-3,11	0
GMFB	-2,42	0
FAM3C	-2,31	0
C6orf115	-2,09	0
CBFB	-2,08	0
SWAP70	-2,00	0
C5orf15	-2,03	0
PSAT1	-2,00	0,001

A threshold of 2-fold and q-value <0.05 (adjusted p-value) was used to generate lists of differently expressed genes.

Supplementary Table S4b

Downregulated genes common for electroporation and lipofectamine

Electroporation

Gene symbol	Fold change	q-value
TMEM9B	-3,13	0
FSCN1	-3,11	0
GMFB	-2,42	0
FAM3C	-2,31	0
CBFB	-2,08	0
SWAP70	-2,00	0
PSAT1	-2,00	0,001

Liposomal transfection

Gene symbol	Fold change	q-value
TMEM9B	-2,07	0,012
FSCN1	-4,44	0
GMFB	-2,35	0,003
FAM3C	-2,25	0,006
CBFB	-2,11	0,010
SWAP70	-2,29	0,005
PSAT1	-2,73	0,001

A threshold of 2-fold and q-value <0.05 (adjusted p-value) was used to generate lists of differently expressed genes.

Supplementary Table S5

Sequence alignment of miR-145 with miR-642a, miR-331 and miR-140 (5'→3')

```
GUCCAGUUUUCCCAGGAAUCCCU      (miR-145)
GUCCCUCUCCAAAUGUGUCUUG       (miR-642a)

GUCCAGUUUUCCCAGGAAUCCCU      (miR-145)
CUAGGUAUGGUCCCAGGGAUCC       (miR-331)

GUCCAGUUUUCCCAGGAAUCCCU      (mir-145)
CAGUGGUUUUACCCUAUGGUAG       (mir-140)
```

Supplementary Table S2

Upregulated and downregulated genes in MSCs transfected with smiR-145 using lipofectamine (compared to smiR-neg control)

A threshold of 2-fold and q-value <0.05 (adjusted p-value) was used to generate lists with differently expressed genes

Gene	Fold Change	q-value	Gene	Fold Change	q-value
CXCL10	166.5	0	ACO1	-11.6	0
TNFSF10	98.2	0	AKR1B10	-8.6	0
IFIT2	73.1	0	IFI6	-7.8	0
OASL	68.3	0	NT5DC2	-5.5	0
CCL5	58.2	0	PLAU	-5.2	0
HERC5	56.3	0	GLS	-4.5	1.79E-04
RSAD2	51.2	0	GDF15	-4.5	1.79E-04
CCL5	49.7	0	FSCN1	-4.4	1.79E-04
ISG20	43.2	0	ARHGDIB	-4.3	1.79E-04
IFNB1	35.1	0	TXNDC12	-3.9	1.79E-04
CXCL11	34.2	0	KIAA0114	-3.7	1.79E-04
IFIT3	34.1	0	GPR68	-3.7	1.79E-04
GBP4	31.8	0	TMEM119	-3.7	1.79E-04
IFIT3	31.2	0	PAMR1	-3.5	2.50E-04
IL18BP	30.2	0	CDKN2B	-3.5	2.50E-04
IFIH1	29.4	0	RAB3IL1	-3.2	2.50E-04
IFI27	28.9	0	WRB	-3.2	2.50E-04
TNFSF13B	28.6	0	PRKCA	-3.1	2.50E-04
MX2	24.9	4.31E-05	CERK	-3.1	2.50E-04
TNFSF13B	24.0	4.31E-05	LRRC17	-3.1	4.00E-04
OAS3	20.5	4.31E-05	CMBL	-3.1	4.00E-04
CCL8	19.9	4.31E-05	RPS23	-3.1	4.00E-04
BST2	19.5	4.31E-05	CDKN2B	-3.1	6.25E-04
OAS1	18.3	4.31E-05	KRT10	-3.0	4.00E-04
DDX58	18.1	4.31E-05	CXCL6	-3.0	0.001410256
OASL	17.9	4.31E-05	FAP	-3.0	9.09E-04
OAS2	17.6	4.31E-05	HNRPA1P4	-2.9	5.56E-04
MX1	17.5	4.31E-05	DLEU1	-2.9	5.56E-04
IFIT1	17.0	4.31E-05	MTSS1	-2.9	0.001861702
IDO1	16.7	4.31E-05	MAP4K4	-2.9	6.67E-04
GBP1	16.6	4.31E-05	CERK	-2.8	6.67E-04
INDO	16.1	4.31E-05	AKR1C2	-2.8	0.002541667
GBP1	15.9	4.31E-05	ODC1	-2.8	8.87E-04
CFB	15.9	4.31E-05	MAP4K4	-2.8	9.09E-04
OAS1	15.9	4.31E-05	C5orf21	-2.7	9.56E-04
OAS2	15.5	4.31E-05	PSAT1	-2.7	0.001351351
ISG15	15.0	4.31E-05	UBE2C	-2.7	0.00297619
IFIT1	14.9	4.31E-05	CLDN11	-2.7	0.002366071
IFI44L	14.6	4.31E-05	LOC643873	-2.7	0.001214286
HES4	14.4	4.31E-05	EIF4B	-2.7	0.00125
KIF5C	13.5	4.31E-05	HNRPA1L-2	-2.7	0.001428571
LGALS9	13.0	4.31E-05	C20orf100	-2.7	0.002107843
OAS1	13.0	4.31E-05	GEM	-2.7	0.001666667
GBP5	12.7	4.31E-05	LOC729779	-2.7	0.001381579
IFITM1	12.5	4.31E-05	PBK	-2.7	0.00297619
HERC6	12.5	4.31E-05	ZMAT3	-2.6	0.002107843
CMPK2	12.3	4.31E-05	WNT5A	-2.6	0.002366071
IRF7	12.2	4.31E-05	BEND6	-2.6	0.001428571
SLC15A3	12.2	4.31E-05	LOC400455	-2.6	0.001647727
EPSTI1	12.1	4.31E-05	NAP1L1	-2.6	0.001569767

LOC100129681	12.1	4.31E-05		TSPAN6	-2.6	0.001847826
USP18	11.8	4.31E-05		ALDH1A3	-2.6	0.001428571
RTP4	11.8	4.31E-05		FAM172A	-2.6	0.001875
IFI6	11.5	4.31E-05		FAM173B	-2.6	0.002163462
IRF7	11.0	4.31E-05		HMOX1	-2.6	0.002366071
PRIC285	10.8	4.31E-05		PCYOX1	-2.6	0.002541667
RARRES3	10.8	4.31E-05		IARS	-2.6	0.002366071
CKB	10.5	4.31E-05		C18orf55	-2.6	0.002040816
AIM2	10.0	7.25E-05		MLPH	-2.5	0.004077381
SAMD9	9.7	7.25E-05		GLT8D2	-2.5	0.00359589
SAMD9L	9.7	7.25E-05		FAM171A1	-2.5	0.002541667
PARP14	9.6	7.25E-05		LOC646688	-2.5	0.003115385
LOC100128274	9.5	7.25E-05		LOC388275	-2.5	0.0037
ZC3HAV1	9.4	7.25E-05		HNRNPA0	-2.5	0.002745902
CASP1	9.4	7.25E-05	-	OLFML2A	-2.5	0.006651376
CASP1	9.4	7.25E-05		LXN	-2.5	0.00494382
HLA-F	9.3	7.25E-05		LPXN	-2.5	0.003506944
LAP3	9.2	7.25E-05		PYCR1	-2.5	0.003085938
IFI35	8.8	1.28E-04		VAT1	-2.5	0.003963415
KIAA1618	8.7	7.25E-05		C7orf41	-2.5	0.003506944
HIST2H2AA4	8.3	1.28E-04		MYH10	-2.5	0.004066265
HIST2H2AA3	8.3	1.28E-04		LOC100129673	-2.4	0.00375
XAF1	8.3	1.56E-04		TGFBR2	-2.4	0.003432836
GCH1	8.2	1.28E-04		FAM43A	-2.4	0.003219697
TYMP	8.2	1.28E-04		DTD1	-2.4	0.003506944
TYMP	8.2	1.28E-04		PCYOX1	-2.4	0.003963415
GJD3	8.1	1.28E-04		ALDH1A3	-2.4	0.003455882
PARP12	8.0	1.28E-04		KCNMA1	-2.4	0.004738372
BATF2	7.9	1.28E-04		CCDC34	-2.4	0.003506944
PARP9	7.8	1.56E-04		PGD	-2.4	0.004744318
PSMB9	7.7	2.08E-04		TACC2	-2.4	0.003682432
ECGF1	7.5	2.15E-04		LOC649049	-2.4	0.003963415
WARS	7.5	2.08E-04		APPL2	-2.4	0.003963415
CLDN23	7.4	2.15E-04		LOC730107	-2.4	0.003963415
LOC389386	7.3	1.83E-04		TOP2A	-2.4	0.0081875
APOBEC3G	7.3	1.83E-04		IMPDH2	-2.4	0.006651376
PLEKHA4	7.2	2.15E-04		CMTM8	-2.4	0.004741379
TAP1	7.2	2.15E-04		TOX2	-2.4	0.005241935
IFI44	7.2	2.15E-04		LOC392437	2.4	0.005833333
HIST2H2AC	7.0	2.15E-04		GMFB	-2.4	0.003963415
APOBEC3G	7.0	2.15E-04		RPLP0	-2.3	0.005083333
DDX60L	7.0	2.15E-04		LOC729423	-2.3	0.005241935
IFIT3	6.9	2.81E-04		LOC642741	-2.3	0.006504854
TAP2	6.9	2.15E-04		SLC14A1	-2.3	0.005850515
CXCL16	6.9	4.01E-04		CD59	-2.3	0.006651376
WARS	6.6	2.60E-04		CXCL6	-2.3	0.009392857
XAF1	6.6	4.42E-04		CXCL1	-2.3	0.008955224
DHX58	6.5	3.03E-04		TTC3	-2.3	0.005526316
MYD88	6.5	2.37E-04		PIR	-2.3	0.006504854
CCL2	6.5	2.37E-04		POPDC3	-2.3	0.00510989
LOC400759	6.3	2.81E-04		SWAP70	-2.3	0.005319149
NCOA7	6.3	3.19E-04		ODZ4	-2.3	0.010098684
SP110	6.2	3.19E-04		EIF3L	-2.3	0.006464646
STX11	6.1	3.19E-04		MGP	-2.3	0.008778195
PARP9	6.1	4.09E-04		BAX	-2.3	0.006651376
TLR3	6.1	3.33E-04		UBE2C	-2.3	0.009827586
TRIM21	6.1	3.33E-04		TRIB3	-2.3	0.006464646
PLSCR1	6.0	3.33E-04		C17orf45	-2.3	0.007146018
TMEM140	5.9	4.57E-04		E2F7	-2.3	0.006651376

ZMYND15	5.8	4.62E-04	FTH1	-2.3	0.006651376
APOL3	5.8	4.62E-04	DLGAP5	-2.3	0.010400641
HIST2H2AA3	5.8	4.84E-04	FAM3C	-2.3	0.006504854
LOC643384	5.8	4.09E-04	MGAT4B	-2.3	0.006909091
HLA-B	5.8	4.42E-04	SH3PXD2A	-2.2	0.00822314
GMPR	5.7	4.09E-04	WRB	-2.2	0.006504854
SP110	5.7	4.42E-04	KCTD12	-2.2	0.010098684
IL29	5.7	4.09E-04	F2R	-2.2	0.00726087
ECGF1	5.7	4.62E-04	C7orf10	-2.2	0.011691617
FBXO6	5.6	4.62E-04	LOC100129550	-2.2	0.007117117
ZNFX1	5.5	4.67E-04	CDCA3	-2.2	0.010211039
GBP2	5.5	4.67E-04	NMB	-2.2	0.014243902
C19orf66	5.4	4.67E-04	TCTEX1D2	-2.2	0.007456897
TDRD7	5.4	4.57E-04	KLHL5	-2.2	0.008067227
PARP10	5.3	5.20E-04	SNHG9	-2.2	0.007146018
TRIM22	5.3	6.69E-04	LOC642357	-2.2	0.008962963
PPM1K	5.3	4.67E-04	LOC100133328	-2.2	0.009251825
ASPHD2	5.1	6.73E-04	RPL15	-2.2	0.007987288
MLKL	5.1	5.75E-04	EIF3L	-2.2	0.008352713
ZC3HAV1	5.1	6.73E-04	RPL10A	-2.2	0.008352713
NT5C3	5.1	6.73E-04	UBE4B	-2.2	0.00758547
HRASLS2	5.1	9.93E-04	RPS6P1	-2.2	0.008352713
CD74	5.1	9.93E-04	GSTA4	-2.2	0.00726087
TAP2	5.0	8.58E-04	LOC392437	-2.2	0.008560606
UBA7	5.0	8.02E-04	GREM2	-2.2	0.009827586
HLA-E	5.0	7.33E-04	MFAP5	-2.2	0.01492823
TAC3	5.0	0.001192053	DLGAP5	-2.2	0.012472527
NT5C3	5.0	7.25E-04	ARL6IP5	-2.2	0.008352713
PNPT1	4.9	7.33E-04	SVIL	-2.2	0.008352713
RNF213	4.9	8.58E-04	PMEPA1	-2.2	0.008352713
TRIM5	4.8	8.52E-04	GPSM2	-2.2	0.013389175
UBE2L6	4.8	9.89E-04	C9orf119	-2.2	0.008560606
TMEM62	4.7	9.24E-04	LRRC17	-2.2	0.010370968
PARP10	4.7	0.001034483	ICAM3	-2.2	0.008560606
C15orf48	4.7	0.001403226	CDKN3	-2.2	0.014855769
STAT1	4.7	0.001073826	TMEM204	-2.2	0.009392857
UNC93B1	4.6	0.001031469	LOC647276	-2.2	0.013346354
PARP10	4.6	0.001054422	PSPH	-2.2	0.009347826
HCP5	4.6	0.001820809	RPL23	-2.2	0.009827586
GCH1	4.6	0.001003521	C6orf85	-2.2	0.012472527
CD38	4.6	0.00183908	RPL4	-2.2	0.010211039
RASGRP3	4.5	0.001034483	GNPDA1	-2.2	0.008352713
SEPT4	4.5	0.001054422	SHISA2	-2.2	0.011763006
PMAIP1	4.4	0.001073826	SH3D19	-2.2	0.008352713
HLA-F	4.4	0.004090909	FAM129A	-2.2	0.009751773
SP110	4.4	0.001133333	NTN4	-2.1	0.010461783
CD74	4.4	0.001575758	EEF1A1	-2.1	0.009914966
HLA-H	4.3	0.002261905	RPL37A	-2.1	0.010660377
KCTD14	4.3	0.001200658	RPL17	-2.1	0.00989726
APOBEC3F	4.2	0.001233766	CDC20	-2.1	0.016860987
SERPING1	4.2	0.002769608	CCNB2	-2.1	0.018877119
HLA-C	4.2	0.001433121	FHOD1	-2.1	0.009080882
OAS2	4.2	0.001575758	LOC642934	-2.1	0.011370482
HIST2H2BE	4.2	0.002346154	PTPN11	-2.1	0.011729651
APOL1	4.2	0.001455696	SIRPA	-2.1	0.009827586
LYSMD2	4.1	0.001426282	CMTM4	-2.1	0.010016667
NFE2L3	4.1	0.001493711	SLC16A6	-2.1	0.012472527
SLC25A28	4.1	0.0015	CBFB	-2.1	0.010016667
STAT1	4.1	0.001864407	MRPL51	-2.1	0.010016667

APOL2	4.1	0.001575758	LOC728492	-2.1	0.011729651
USP41	4.0	0.001568323	RBPMS2	-2.1	0.016440092
RSPO3	4.0	0.00206044	RPL3	-2.1	0.013115183
IRF1	4.0	0.001686391	EVI2A	-2.1	0.010632911
MSX1	4.0	0.001616766	FNBP1	-2.1	0.010740741
ARL9	3.9	0.001616766	IL8	-2.1	0.011729651
EIF2AK2	3.9	0.001787791	NDP	-2.1	0.017059471
CD68	3.9	0.002865854	LOC645387	-2.1	0.011960227
TRIM38	3.9	0.001864407	UHRF1	-2.1	0.018474026
PHF15	3.9	0.001783626	BZW2	-2.1	0.013085106
CTNNBL1	3.9	0.001622024	TGFBR2	-2.1	0.010945122
MDK	3.9	0.002053073	ITGAE	-2.1	0.011318182
TRIM5	3.9	0.001783626	C18orf55	-2.1	0.010751534
CCL7	3.8	0.003220721	TMEM5	-2.1	0.010740741
LGALS9	3.8	0.00206044	FAM89B	-2.1	0.010740741
DDX60	3.8	0.002261905	NPM3	-2.1	0.012472527
PSMB8	3.8	0.001924157	SUMF1	-2.1	0.012513587
KRTAP1-5	3.8	0.001864407	LOC647030	-2.1	0.011867816
HCG4	3.8	0.007716049	HJURP	-2.1	0.01745614
MDK	3.8	0.002346154	TMEM9B	-2.1	0.012161017
BDKRB1	3.8	0.003650628	C18orf56	-2.1	0.013085106
LOC728946	3.7	0.002261905	LOC158345	-2.1	0.015981308
GCA	3.7	0.002131148	NDFIP2	-2.1	0.011729651
PSMB8	3.7	0.002055556	C16orf53	-2.1	0.014002525
STAT2	3.7	0.002261905	CMTM4	-2.1	0.011960227
EHD4	3.6	0.002261905	AGBL5	-2.1	0.014243902
CFLAR	3.6	0.002939815	ARPC1A	-2.1	0.013085106
LOC389386	3.6	0.002261905	GCLM	-2.1	0.012513587
PSMB8	3.6	0.002342932	PTPN13	-2.1	0.013085106
MLKL	3.6	0.002302632	GALNT11	-2.1	0.011729651
PSME2	3.5	0.002346154	LOC100131940	-2.1	0.013115183
IL4I1	3.5	0.002461735	RPL13A	-2.1	0.013115183
PANX1	3.5	0.00234375	FOXRED2	-2.0	0.014243902
MICB	3.5	0.002512563	LOC729926	-2.0	0.016869469
IFI16	3.5	0.002474619	CC2D2A	-2.0	0.014243902
UBE2L6	3.5	0.002939815	LAGE3	-2.0	0.013718274
C1R	3.5	0.003373894	KDELR3	-2.0	0.015797619
TLR3	3.5	0.0025375	HSBP1	-2.0	0.013389175
LGMN	3.5	0.003650628	EPHX1	-2.0	0.015868545
RHBDF2	3.5	0.002512563	MOCS2	-2.0	0.013718274
LGALS3BP	3.5	0.002939815	FAM45A	-2.0	0.015849057
C1R	3.5	0.003506494	LOC645638	-2.0	0.02219697
KRT34	3.5	0.002769608	NIPSNAP1	-2.0	0.014243902
LOC100133019	3.4	0.00288835	CTHRC1	-2.0	0.019989583
APOL2	3.4	0.002636816	FMNL2	-2.0	0.022817164
PHACTR4	3.4	0.002660891	CORO2B	-2.0	0.016337209
ADAR	3.4	0.002939815	MGP	-2.0	0.02728013
NEFM	3.4	0.004137597	NBL1	-2.0	0.018766094
BTN3A3	3.4	0.003038991	TXNRD1	-2.0	0.016869469
C4orf33	3.4	0.002939815	ABCE1	-2.0	0.014243902
PSMB10	3.4	0.002939815	BRI3BP	-2.0	0.013718274
C6orf192	3.4	0.002939815	EIF3F	-2.0	0.016440092
NUB1	3.4	0.002939815	FAM3C	-2.0	0.014243902
XRN1	3.4	0.002939815	FAM64A	-2.0	0.032875354
TRAFD1	3.3	0.002939815	MLLT11	-2.0	0.014660194
CH25H	3.3	0.003482533	CCNG1	-2.0	0.015849057
UNC93B1	3.3	0.003045455	AGPAT9	-2.0	0.016869469
GSDMD	3.3	0.003045455	LOC641814	-2.0	0.02219697
GAL3ST4	3.3	0.002995392	LOC641814	-2.0	0.02219697

OPTN	3.3	0.003373894
PML	3.3	0.00321267
C10orf10	3.3	0.003816872
CD68	3.3	0.003791322
ACSL5	3.2	0.003650628
LOC654346	3.2	0.003551502
SP100	3.2	0.003340807
APOBEC3F	3.2	0.003551502
SP110	3.2	0.003480176
LGMN	3.2	0.005008993
LRRN3	3.2	0.004023904
LOC285296	3.2	0.003359375
HLA-DRA	3.2	0.006064189
SLC16A4	3.2	0.00565331
TRIM25	3.2	0.003650628
HLA-A29.1	3.2	0.01723382
MOV10	3.2	0.00348913
IL7R	3.2	0.006059322
ADAR	3.2	0.003650628
OGFR	3.2	0.003482533
FLJ21986	3.1	0.005
LBA1	3.1	0.003941532
MIR155HG	3.1	0.003682573
LOC730743	3.1	0.004581784
LRRN3	3.1	0.003941532
RAB8B	3.1	0.003677083
BIRC3	3.1	0.004189189
STAT1	3.1	0.004224138
SLC2A12	3.1	0.003650628
LOC728255	3.1	0.004530075
HK2	3.1	0.004127451
MLKL	3.0	0.003903689
RNF114	3.0	0.003941532
MASTL	3.0	0.003945783
JAK2	3.0	0.004137597
LGALS9B	3.0	0.004601852
MASTL	3.0	0.003941532
BAMBI	3.0	0.004023904
CARD16	3.0	0.004221154
GNB4	3.0	0.004137597
MT1M	3.0	0.005292553
MMP13	3.0	0.004640221
IL33	3.0	0.028560359
DUSP19	3.0	0.004581784
PFKFB4	3.0	0.004127451
RBMS2	3.0	0.004531835
RNF114	3.0	0.004515209
ADARB1	3.0	0.00611204
CD68	3.0	0.005519366
HIST1H2BD	3.0	0.005945017
C5orf41	2.9	0.005125448
GCH1	2.9	0.004530075
BTN3A1	2.9	0.005318021
TNS1	2.9	0.004417939
PLA1A	2.9	0.004641544
PHF11	2.9	0.004863139
ZC3H12A	2.9	0.009125364
PHF11	2.9	0.00525
ELMO2	2.9	0.004530075

TMEM171	2.9	0.004890909
SLC2A5	2.9	0.013182957
ODF3B	2.9	0.005646853
RP2	2.9	0.004936594
SLC6A9	2.9	0.005976027
RHBDF2	2.9	0.004826007
SCO2	2.9	0.005292553
SOD2	2.9	0.025617444
HLA-H	2.9	0.023781475
RBM43	2.9	0.005761246
LY6E	2.8	0.006737013
HIST1H2AC	2.8	0.019658416
BTC	2.8	0.006158333
VCAM1	2.8	0.034578267
LAG3	2.8	0.005761246
VAMP5	2.8	0.005646853
TNFAIP3	2.8	0.007631579
LOC100134073	2.8	0.009993036
KRTAP1-3	2.8	0.00611204
PDCD1LG2	2.8	0.005862069
PATL1	2.8	0.005981229
PDE4B	2.8	0.006028912
LOC728946	2.8	0.006672131
ERAP2	2.8	0.020573218
PNPT1	2.8	0.006737013
SOD2	2.8	0.027360642
IL7R	2.7	0.014738889
NKX3-1	2.7	0.006672131
LOC653610	2.7	0.008442136
IFITM2	2.7	0.007198413
FTSJD2	2.7	0.006258278
DUSP5	2.7	0.00687299
NT5E	2.7	0.006737013
ETV7	2.7	0.006102694
DHRS9	2.7	0.028522167
BTN3A2	2.7	0.013046482
EDN1	2.7	0.009390935
CHEK2	2.7	0.016150964
AGRN	2.7	0.006870968
IFITM3	2.7	0.008192771
GRIP2	2.7	0.006361386
LOC100134304	2.7	0.019116766
C5orf41	2.7	0.007238924
ELF1	2.7	0.006899038
MOBKL2C	2.7	0.006820388
IFIT5	2.6	0.007124601
MUC1	2.6	0.007421136
LOC730996	2.6	0.007631579
SLC16A4	2.6	0.010586253
TGM2	2.6	0.007865854
YEATS2	2.6	0.007198413
C1QTNF1	2.6	0.019075
B3GNT2	2.6	0.007437107
LYPD1	2.6	0.013776596
PTN	2.6	0.008115502
HLA-A	2.6	0.01078
APOL6	2.6	0.007631579
AIDA	2.6	0.007631579
BCL2L13	2.6	0.007631579

PVRL2	2.6	0.007822086
RNF19B	2.6	0.007792308
TXNIP	2.6	0.008159091
CGNL1	2.6	0.008442136
LYPD1	2.6	0.014363426
IL7	2.6	0.009390935
LGALS8	2.6	0.007865854
ITGA2	2.5	0.008720588
NLRC5	2.5	0.008192771
RFX5	2.5	0.008285928
ADARB1	2.5	0.014709821
STAT3	2.5	0.008285928
SECTM1	2.5	0.008720588
CXCL9	2.5	0.009317529
PSMB9	2.5	0.010586253
LOC653631	2.5	0.008442136
HIST1H2BD	2.5	0.011361842
IL15	2.5	0.009598592
TMEM173	2.5	0.008841642
DCP1A	2.5	0.008720588
RNF149	2.5	0.010873016
MUC1	2.5	0.009317529
RBCK1	2.5	0.009317529
ARID5B	2.5	0.010096685
LOC653308	2.5	0.009135174
B4GALT5	2.5	0.009125364
GBP3	2.5	0.009390935
LOC390557	2.5	0.014917401
CHEK2	2.5	0.022642202
TLE4	2.5	0.009548023
LOC728951	2.5	0.009390935
N4BP1	2.5	0.010676944
C3orf38	2.5	0.009317529
RNF122	2.5	0.009390935
PGAM1	2.5	0.009859944
TRIM26	2.5	0.009796348
LOC730996	2.4	0.009867318
PSME1	2.4	0.010178571
NMI	2.4	0.012538071
FAM46A	2.4	0.013776596
C1S	2.4	0.029593949
TM4SF4	2.4	0.025310315
IRF9	2.4	0.012645202
CD274	2.4	0.010497275
SLFN12	2.4	0.010096685
RAB20	2.4	0.010096685
LOC728956	2.4	0.01030137
FAM111A	2.4	0.010165289
IL10	2.4	0.012139175
HLA-G	2.4	0.039740634
C3orf38	2.4	0.01045082
BAZ1A	2.4	0.011062005
LOC285047	2.4	0.010586253
PVRL2	2.4	0.010586253
SIGIRR	2.4	0.010873016
LOC147645	2.4	0.01078
C6orf188	2.4	0.010676944
CCDC109B	2.4	0.010873016
REC8	2.4	0.013776596

FAM111A	2.4	0.011364829
PLEKHH1	2.4	0.011914063
IL12A	2.4	0.012468112
SERTAD1	2.4	0.01138089
FST	2.4	0.013644802
TMEM189-	2.4	0.012139175
ATOH8	2.4	0.012538071
PML	2.4	0.011436031
RASD2	2.4	0.015393013
IL15	2.4	0.012139175
STOM	2.4	0.014230769
PGAM4	2.3	0.01230179
PPFIA4	2.3	0.01230179
ESM1	2.3	0.043651961
FSTL1	2.3	0.014561503
CYP2J2	2.3	0.014363426
NBN	2.3	0.012172237
FAM65B	2.3	0.023852679
SCARB2	2.3	0.012139175
HIAT1	2.3	0.013557214
FGF2	2.3	0.016703586
SFRP1	2.3	0.024916667
HAVCR2	2.3	0.012575949
TRIM69	2.3	0.013776596
C1S	2.3	0.0334
CENTA1	2.3	0.021174242
MTMR11	2.3	0.01302267
RBCK1	2.3	0.013648148
HLA-DPA1	2.3	0.027360642
NAMPT	2.3	0.014396552
SMARCA5	2.3	0.013260599
MAFB	2.3	0.013776596
LGALS8	2.3	0.013776596
CCND3	2.3	0.01323125
RORA	2.3	0.013762255
WSB1	2.3	0.0149117
STOM	2.3	0.01841785
PML	2.3	0.013579404
LOC100129566	2.3	0.013762255
ZNF702P	2.3	0.013776596
IRF7	2.3	0.014396552
B4GALT4	2.3	0.014805987
SPATA13	2.3	0.013776596
EFHD1	2.3	0.0372
LOC732371	2.3	0.013776596
LOC730052	2.3	0.01421729
RPS6KC1	2.3	0.013879108
TRIM5	2.3	0.013776596
DYNLT1	2.3	0.013776596
IFI30	2.3	0.027909699
ODF2L	2.3	0.01440367
PSMA4	2.3	0.013776596
STAT6	2.3	0.013776596
ADPRHL2	2.3	0.013762255
SNRPC	2.3	0.013776596
PTGS2	2.3	0.046594945
C19orf28	2.3	0.013814858
BMPR2	2.3	0.013776596
NFKBIA	2.3	0.020627395

PRKD1	2.3	0.014709821
DENND1A	2.3	0.01421729
C3AR1	2.3	0.013847059
HAS1	2.3	0.032449219
FST	2.2	0.018777666
USP42	2.2	0.014396552
ZFYVE20	2.2	0.014738889
GPR180	2.2	0.014690315
GNB4	2.2	0.014485126
CTSS	2.2	0.037602941
RBMS2P	2.2	0.014642045
LOC100133012	2.2	0.030416013
PGAM4	2.2	0.014690315
LOC645634	2.2	0.014503425
C19orf28	2.2	0.014709821
ALPK1	2.2	0.014643665
ANKIB1	2.2	0.014709821
LIPA	2.2	0.014643665
LOC100133489	2.2	0.03948913
ACSL5	2.2	0.015863931
PROCR	2.2	0.018103272
INSIG1	2.2	0.01511488
AXUD1	2.2	0.01488385
GNA13	2.2	0.016353945
C7orf42	2.2	0.01511488
CXorf38	2.2	0.014961538
OTUD4	2.2	0.015701087
RPS6KC1	2.2	0.015822511
INSIG1	2.2	0.015748373
ANKFY1	2.2	0.016414894
TNFRSF14	2.2	0.01654334
LOC732007	2.2	0.015697168
OAS1	2.2	0.015975216
SPATS2L	2.2	0.016075269
LOC728946	2.2	0.028926256
LOC643384	2.2	0.016314103
PI4K2B	2.2	0.016427813
ZFP36L2	2.2	0.017942387
APOBEC3D	2.2	0.016150964
ZNF350	2.2	0.017525988
PDGFRL	2.2	0.021174242
FLT3LG	2.2	0.016959034
SOCS1	2.2	0.017639463
SQRDL	2.2	0.019568452
LOC255620	2.2	0.016525424
CCRL1	2.2	0.021365348
SIX1	2.2	0.017369792
B2M	2.2	0.018084016
JUNB	2.2	0.029593949
CHMP5	2.2	0.017942387
TRIM56	2.2	0.017201883
RGS20	2.2	0.043150492
MCL1	2.2	0.020639313
CD82	2.1	0.022009259
SAMHD1	2.1	0.018649194
EID3	2.1	0.020605769
WSB1	2.1	0.018401222
SOCS2	2.1	0.020427308
KIAA1751	2.1	0.040046695

BNIP3	2.1	0.017614108
CEBPD	2.1	0.022051756
TSKU	2.1	0.018362245
PSMA4	2.1	0.0176294
MOCOS	2.1	0.021957328
WSB1	2.1	0.020627395
RAB43	2.1	0.01841785
PSMF1	2.1	0.018084016
CAPN3	2.1	0.022642202
H1F0	2.1	0.022974453
USF1	2.1	0.01944831
PYCARD	2.1	0.018649194
KLF6	2.1	0.020497104
IL15RA	2.1	0.020455882
COMMD10	2.1	0.018992986
ZFYVE26	2.1	0.018649194
RABGAP1L	2.1	0.018825301
GRINA	2.1	0.020931559
WISP1	2.1	0.030970266
PCDH17	2.1	0.020497104
SCML1	2.1	0.019881423
TAPBP	2.1	0.02185514
BMPR2	2.1	0.019143426
STAT3	2.1	0.020187008
PDZD2	2.1	0.027360642
SNORD89	2.1	0.029593949
GADD45B	2.1	0.020497104
SYNM	2.1	0.020497104
LIMA1	2.1	0.020497104
TRADD	2.1	0.020044379
L2HGDH	2.1	0.020497104
SLC30A1	2.1	0.020639313
PCGF5	2.1	0.020814286
SPPL2A	2.1	0.023903197
GCNT1	2.1	0.042553041
SLC39A14	2.1	0.046248285
BDNF	2.1	0.023601083
MYCBP2	2.1	0.021474719
LOC100130308	2.1	0.020497104
C12orf31	2.1	0.020497104
NAMPT	2.1	0.027981728
KCNS3	2.1	0.027285959
PGAM4	2.1	0.021365348
NAPA	2.1	0.021474719
PRKD2	2.1	0.021908752
PML	2.1	0.021861007
TJP1	2.0	0.021423872
RGMB	2.0	0.024766725
LOXL3	2.0	0.025568576
FAM125B	2.0	0.021365348
BTN3A2	2.0	0.023053539
GRINA	2.0	0.022449262
CX3CL1	2.0	0.036451493
PPP2R2A	2.0	0.021957328
MCL1	2.0	0.022642202
ETV3	2.0	0.029601106
SERPINB1	2.0	0.023031818
ATP8B4	2.0	0.048101351
SP100	2.0	0.023852679

GBP6	2.0	0.023852679
TINF2	2.0	0.022783883
IL15RA	2.0	0.023903197
GTF2B	2.0	0.022966179
MR1	2.0	0.02478471
ACOT7	2.0	0.023442029
LAYN	2.0	0.023601083
PIM1	2.0	0.027360642
PGAM1	2.0	0.023031818
HLA-DOB	2.0	0.024598057
P2RY5	2.0	0.028522167
TRAF3IP2	2.0	0.03948913
DNAJA1	2.0	0.024233156
LOC100131091	2.0	0.023657658
RAD9A	2.0	0.023852679
MAT2B	2.0	0.027360642