2009

TOWARDS AN IN VITRO MODEL FOR RNA INTERFERENCE-MEDIATED SUPPRESSION OF S100A8

Rudha M. Al-Rohani
Western University

TOWARDS AN *IN VITRO* MODEL FOR RNA INTERFERENCE-MEDIATED SUPPRESSION OF S100A8

(Spine title: Towards an *In Vitro* Model for the Suppression of S100A8)

(Format: Monograph)

by

Rudha M. Al-Rohani
B.Sc. (Honors Biology)

Department of Biochemistry

Submitted in partial fulfillment
of the requirements for the degree of

Master of Science

School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada
August 2009

THE UNIVERSITY OF WESTERN ONTARIO
SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

CERTIFICATE OF EXAMINATION

Supervisor	Examination Board
Dr. Richard Rozmahel	Dr. Gary Shaw
Supervisory Committee	Dr. Brian Shilton
Dr. Gary Shaw	Dr. Shiva Singh
Dr. Jim Koropatnick	

The thesis by

Rudha M. Al-Rohani

entitled:

Towards an *in vitro* model for RNA interference-mediated suppression of S100A8

is accepted in partial fulfillment of the
requirements for the degree of Master of Science

Date _____

_____
Chair of Examination Board

ABSTRACT

The S100 calcium-binding protein A8 (S100A8) has been identified as a potential genetic modifier of the lung disease of cystic fibrosis (CF) patients as well as mice. S100A8 and its heterodimeric partner, S100A9, are expressed by neutrophils and monocytes and secreted to the extracellular milieu during inflammation where they act as potent chemokines for neutrophil recruitment. The aim of this study was to assess the efficacy of short-hairpin RNAs (shRNAs) in suppressing murine S100A8 expression *in vitro* as a preliminary model to the study of S100A8 knockdown in CF mice. Stimulation of murine macrophage-like P388D1 and granulocytic 32D cells failed to induce S100A8 expression. COS-7 (African green monkey kidney) cells, stably transfected with murine S100A8, showed S100A8 mRNA but no protein. Introduction of murine S100A9 into wild-type and COS-7 cells stable for S100A8 expression revealed S100A9, but no S100A8, protein. The fact that S100A8 protein was not detected contradicts, at least, the idea that S100A9 protein requires S100A8 for stability, although the opposite may also be true. Three different miR-30-based shRNAs targeting S100A8 and a non-specific shRNA (control) were evaluated for their knockdown efficacy. Two of the three hairpins tested (shRNA_mA8_65 and shRNA_mA8_250) were able to significantly reduce S100A8 mRNA levels compared the untreated and control-treated cells (p=0.001 and p=0.033, respectively), achieving 62% and 25% suppression, respectively. This study has identified shRNAs that can specifically silence the expression (and achieve stable knockdown) of S100A8 in COS-7 cells over-expressing the S100A8 gene. This will be valuable for both *in vitro* and *in vivo* functional studies of S100A8; particularly, to study the effects of S100A8 suppression on the inflammatory lung phenotype in CF mice.

KEYWORDS

Cystic fibrosis (CF), S100A8, MRP8, calgranulin A, calprotectin, lung disease, inflammation, RNA interference (RNAi), short-hairpin RNA (shRNA), mouse models

ACKNOWLEDGEMENTS

I would like to express my immense gratitude to my teachers, colleagues, and friends. I am profoundly indebted to my supervisor, Dr. Richard Rozmahel, for giving me the opportunity to undertake my M.Sc. under his excellent expertise and valuable guidance; I am thankful for his advice, insightful comments and encouragement.

I am also indebted to my supervisory committee members, Dr. Gary Shaw and Dr. Jim Koropatnick, for generously volunteering their time to attend our meetings and for their excellent advice and direction.

I would also like to thank the wonderful members of the Rozmahel lab (past and present), whose advice, assistance and companionship were instrumental in enriching this experience. I extend my thanks and appreciation to Mary Keet for her expert advice and technical assistance throughout this project. I also thank Dr. Zareen Amtul for her guidance in methods related to tissue culture and molecular biology, as well as the pleasant chats we had which made the long days less tedious. I am very thankful to my good friend and colleague, Wei Tang, who was kind, thoughtful and always there to assist me when I needed help. I also would like to thank my good friend and colleague, Evangelos Giakoumatos, for being a great listener and for his ever positive attitude, and not to mention, the delicious home-made Greek pastries that he brought for the lab every now and then. I also thank Lynn Wang for her technical support and cheerful smile. I am also thankful to Dr. Susan Newbigging for her positive attitude and for helping me to adjust and feel comfortable upon my arrival at the Rozmahel lab.

I would like to thank Dr. Michael Golding for his contribution to the design of the shRNAs used in this study and his helpful advice. I am also thankful to my friend, Bashar Hadi, for his help in reproducing the introductory figures in the thesis.

I am especially thankful to my wonderful wife and my little boy for their love, encouragement and patience especially during the writing of this thesis. I also thank my parents for their love and continued support.

Funding from the Lawson Health Research Institute (LHRI) Internal Grant and the Canadian Institute of Health Research (CIHR) supported this study. I would also like to acknowledge the Ontario Government for their financial support (Ontario Graduate Scholarship, 2007-2008) during my M.Sc. candidature.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

32D	murine bone marrow cell line
AA	arachidonic acid
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BM	bone marrow
bp	base pairs
°C	degree Celsius
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
Cl^-	chloride
CLCA3	chloride channel calcium activated
COS-7	African green monkey kidney cells
Cu^{2+}	copper
DAMP	damage-associated molecular pattern
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphate
dsRNA	double-stranded RNA
DTT	dithriothrietol
EDTA	ethylene-diamine-tetraacetate
ENaC	epithelial sodium channel
ER	endoplasmic reticulum
FGF-2	fibroblast growth factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCSF	granulocyte colony-stimulati ng factor
HCO_3^-	bicarbonate
H&E	hematoxylin and eosin stain
HEK293	human embryonic kidney cell line
H_2O_2	hydrogen peroxide
IFN-β	interferon-beta
IFN-γ	interferon-gamma
IL-1β	interleukin-1-beta
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
IRES	internal ribosome entry site
K^+	potassium
kDa	kilo daltons
LPS	lipopolysaccharide
LTR	long terminal repeat

miRNA	microRNA
mRNA	messenger RNA
MRP	migration inhibitory factor-related protein
Na^+	sodium
NADPH	nicotinamide adenine dinucleotide phosphate
NIH3T3	mouse embryonic fibroblast cell line
NF-κB	nuclear factor-kappa B
O_2^-	superoxide anion
ORCC	outwardly rectifying chloride channel
ORF	open reading frame
P388D1	murine macrophage-like cell line
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEI	polyethylenimine
PKA	protein kinase A
PMA	phorbol 12-myristate 13-acetate
Poly(I:C)	polyinosinic acid-polycytidylic acid
Pre-miRNA	precursor miRNA
Pri-miRNA	primary miRNA
RAW 264.7	mouse leukaemic macrophage-like cell line
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RT	reverse-transcriptase
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
shRNA	short-hairpin RNA
siRNA	small interfering RNA
ssRNA	single-stranded RNA
Tg	transgenic
TLR4	Toll-like receptor 4
TNF-α	tumor necrosis factor-alpha
UTR	untranslated region
WT	wild-type
Zn^{2+}	zinc

INTRODUCTION

The aim of the current research in our lab is to identify and characterize genetic modifiers of lung disease in cystic fibrosis mouse models. S100A8 is a member of a large family of calcium-binding proteins implicated in a number of cellular processes, the most notable of which is its role in the inflammatory response in acute and chronic inflammatory conditions, including cystic fibrosis. The objective of this study was to assess the knockdown of S100A8 expression using short-hairpin RNA. This introduction will review the involvement of S100A8 and its interacting partner, S100A9, in cystic fibrosis and discuss their protein and gene structure, heterodimer formation, expression patterns and functions. In addition, this introduction also provides insights on RNA interference, including history, mechanisms and potential applications for silencing gene expression. Background information and rationale behind each section of the thesis is also discussed.

1.1 S100 PROTEINS

The EF-hand family of calcium-modulated signaling proteins is one of four classes of intracellular calcium-binding proteins (Kerkhoff *et al.*, 1998). Members of this family share the structurally-related Ca^{2+}-binding motif (Donato, 2001; Heizmann *et al.*, 2002a; Ravasi *et al.*, 2004) and include proteins such as calmodulin, troponin C, parvalbumin and S100 proteins, which comprise the largest subset within the EF-hand superfamily with more than 20 known members (Heizmann, 1992; Kawasaki *et al.*, 1998; Marenholz *et al.*, 2004).

1.1.1 Discovery of S100 Proteins

Moore (1965) described the first members of the S100 protein family, a mixture of S100A1 and S100B that was purified from bovine brain tissue. They were named "S100" because of their solubility in 100% ammonium sulfate at neutral pH (Moore, 1965). The S100 family members are found exclusively in vertebrates and are evolutionarily related (Ravasi *et al.*, 2004). Figure 1-1 shows the 20 members of the human S100 protein family which are encoded in a cluster on chromosome 1q21.3 (Donato, 2001; Heizmann *et al.*, 2002a; Roth *et al.*, 2003). To date, 19 murine S100 genes have been identified, and of those, 16 are located as a cluster on chromosome 3 (Kawasaki *et al.*, 1998; Ravasi *et al.*, 2004; Ridinger *et al.*, 1998).

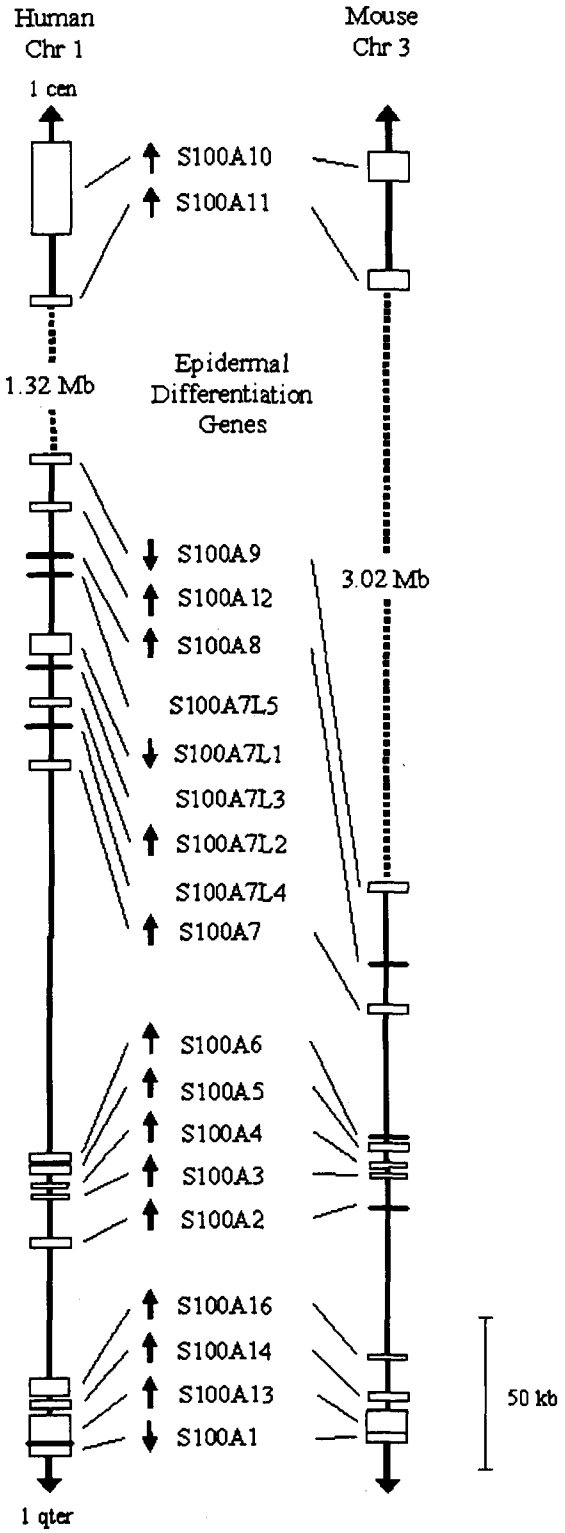

Fig. 1-1. S100 chromosomal clusters. Graphical representation of the S100 clusters on human chromosome 1q21 and mouse chromosome 3. Arrows indicate the direction of the genes from the 5′ to the 3′ UTR. *(Adapted from Marenholz et al., 2004)*

1.1.2 Structure of S100 Proteins

S100 proteins are characterized by a low molecular weight (10-14 kDa) and two calcium-binding motifs of the EF-hand type conformation linked by a central hinge region (Donato, 2001) (Fig. 1-2). The EF hand is a helix-loop-helix structural domain, consisting of two α-helices (E and F) positioned roughly perpendicular to one another and connected by a short loop that binds Ca^{2+} (Donato, 2001, 2003; Mareholz *et al.*, 2004). The C-terminal EF-hand (12 amino acids) contains the classical calcium-binding motif, whereas the N-terminal EF-hand (14 amino acids) is unconventional and characteristic for S100 proteins (Donato, 2001). Each EF-hand can bind one Ca^{2+} ion with the affinity of the C-terminal EF-hand for calcium being 100-fold stronger than that of the N-terminal EF-hand (Heizmann and Cox, 1998; Zimmer *et al.*, 2003). Some members of the S100 family of proteins also bind Zn^{2+} and Cu^{2+} at sites distinct from those that bind Ca^{2+} (Baudier *et al.*, 1986; Donato, 1991; Schafer *et al.*, 2000). The C-terminal domains and the hinge region exhibit the highest sequence (and structural) variation between the proteins (Kligman and Hilt, 1988; Kretsinger, 1987; Lackmann *et al.*, 1993), and are thought to be responsible for the target specificity of each member (Heizmann and Cox, 1998; Kligman and Hilt, 1988).

S100 proteins are likely to exist in cells as dimers, with most forming anti-parallel homodimers, consisting of two identical polypeptides held together by non-covalent bonds (Brodersen *et al.*, 98; Ishikawa *et al.*, 2000; Zimmer *et al.*, 2003). Some S100 proteins may also form heterodimers or higher-order complexes, and this appears to be important for their function (Heizmann, 2002b). The S100A8/S100A9 heterodimer is the most abundant example (Edgeworth *et al.*, 1991; Passey *et al.*, 1999b).

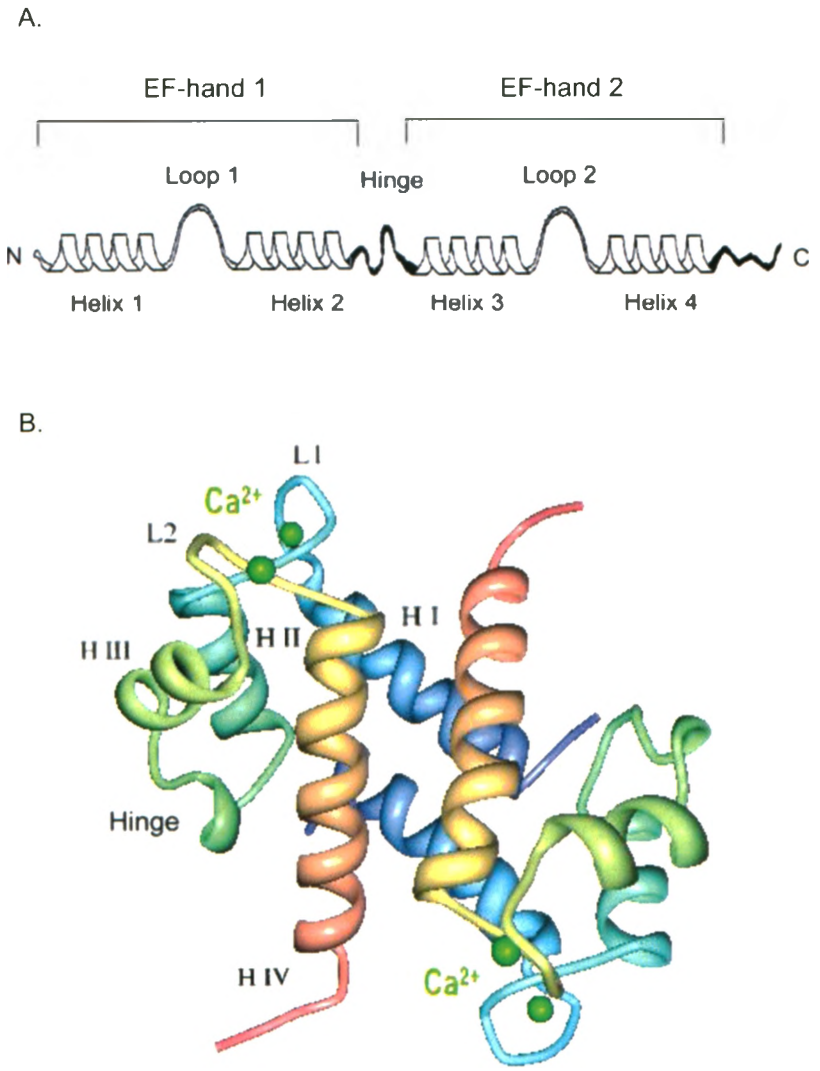

Fig. 1-2. Structure of S100 proteins. (A) Schematic representation of the secondary (helix-loop-helix) structure of S100 proteins; each monomer is composed of two EF-hand Ca^{2+}-binding motifs, connected by a central hinge region *(Adapted from Donato, 2001).* **(B)** Crystal structure of Ca^{2+}-loaded S100A8 homodimer, showing helices (H) I through IV and the hinge region for one monomer. Calcium ions are represented as green dots within the Ca^{2+}-binding loops, L1 and L2. *(Adapted from RCSB Protein Data Bank, http://www.rcsb.org/pdb/explore.do?structureId=1MR8)*

1.1.3 Functions of S100 Proteins

S100 proteins have been shown to have a variety of intracellular and extracellular roles, including regulation of protein phosphorylation, transcription factors, cytoskeleton dynamics, enzyme activity, cell growth and differentiation and the inflammatory response (Donato, 1999, 2001, 2003; Passey *et al.*, 1999b; Schafer and Heizmann, 1996; Zimmer *et al.*, 1995). They regulate biological activity by binding Ca^{2+}, which leads to conformational changes in the protein structure to facilitate interactions with target proteins important in regulating intracellular signaling cascades and activation of transcription factors (Donato, 2001, 2003). Several S100 proteins, including S100B, S100A4, S100A8/S100A9 and S100A12 are also secreted from cells, reflecting possible extracellular functions for these proteins (Donato, 2001, 2003). The mechanism of their secretion, however, remains unclear.

In contrast to calmodulin, the earliest member of the EF-hand superfamily which is ubiquitously expressed (Ravasi *et al.*, 2004), many S100 proteins show cell- and tissue-specific expression patterns, indicative of the specificity of their function (Roth *et al.*, 2003). Some cell-types can also be stimulated to express specific S100 proteins. For example, pro-inflammatory mediators such as LPS, IL-1β and TNF-α have been shown to induce S100A8/S100A9 expression in cultured human keratinocytes (Mork *et al.*, 2003).

S100 proteins are suggested to have a role in numerous diseases including cancer, Down's syndrome, Alzheimer's disease, cystic fibrosis (CF), cardiomyopathy and psoriasis (Heizmann *et al.*, 2002b). A number of S100 proteins have been implicated in cancer progression through specific roles in cell survival and apoptosis pathways (Donato, 2001; Heizmann *et al.*, 2002b; Most *et al.*, 2003). Mutations have been

identified in the S100 gene cluster on chromosome 1q21 in cancer cells, which exhibit altered levels of S100 gene expression compared to normal cells in several cancer types, including breast, colorectal, gastric, prostate, lung and hepatocellular carcinoma (Emberley *et al.*, 2004). S100A8, S100A9 and S100A12 have also been suggested to play important roles in inflammatory conditions, including lung disease in CF.

1.2 S100A8 AND S100A9

1.2.1 Discovery and Characterization

The complex of S100A8 and S100A9 (designated S100A8/S100A9 herein) was originally discovered in 1980 by Fagerhol *et al.* and provisionally named L1 protein. The complex was purified from the cytoplasm of human neutrophils by Dale *et al.* (1983), and later described by Odink *et al.* (1987) as macrophage migration inhibitory factor-related protein (MRP), consisting of MRP-8 and MRP-14, named based on their relative masses of 8 kDa and 14 kDa. The name 'calprotectin' was proposed for the complex to highlight its anti-microbial properties (Steinbakk *et al.*, 1990). It was then discovered that the sequence of MRP-8 was identical to the sequence of the "cystic fibrosis antigen" (Anderson *et al.*, 1988; Wilkinson *et al.*, 1988), a serum protein found to be abnormally elevated in CF patients (Dorin *et al.*, 1987; Fanjul *et al.*, 1995; Renaud *et al.*, 1994). The individual proteins were referred to as 'calgranulin' to indicate the presence of the Ca^{2+}-binding protein in the cytosol of granulocytes (Wilkinson *et al.*, 1988). The more widely used names, S100A8 and S100A9, were adopted in 1995 (Schafer *et al.*, 1995). Murine S100A8 was purified in 1999 as chemotactic protein, CP-10, according to its molecular mass of 10.3 kDa (Lackmann *et al.*, 1993). Table 1-1 summarizes the different names and acronyms that have been assigned to the two proteins over the years.

Table 1-1. Acronyms of S100A8, S100A9 and S100A8/S100A9.

S100A8	Myeloid-related protein 8 (MRP-8), calgranulin A (CAGA), p8, CGLA, cystic fibrosis antigen (CFAg), leukocyte L1 light chain (L1L), 60B8Ag, calprotectin L1L subunit, CP-10 (murine)
S100A9	Myeloid-related protein 14 (MRP-14), calgranulin B (CAGB), p14, CGLB, leukocyte L1 heavy chain (L1H), 60B8AG, calprotectin L1H subunit
S100A8/S100A9 complex	Calprotectin, MRP-8/14, calgranulin A/B, p8/14, L1 protein, p34, 27E10 antigen, cystic fibrosis protein

1.2.2 Gene Structure of S100A8 and S100A9

The S100A8 and S100A9 gene sequences consist of three exons and two introns (Legasse and Clerc, 1988) as shown in Fig. 1-3. The human S100A8 gene contains an open reading frame (ORF) of 279 nucleotides (nt), encoding a protein of 93 amino acids with a predicted molecular mass of 10.8 kDa, whereas human S100A9 contains an ORF of 352 nt, encoding a 114 amino acid peptide with a molecular mass of 13.2 kDa (Edgeworth *et al.*, 1991; Odink *et al.*, 1987). Murine S100A8 (89 amino acids; 10.3 kDa) and S100A9 (113 amino acids; 13.0 kDa) were cloned by Lagasse and Weissman in 1992. A comparison of the mouse and human S100A8 and S100A9 cDNAs reveals nucleotide identities of 64% and 59%, respectively (Lagasse and Weissman, 1992). At the protein level, both mouse S100A8 and S100A9 share 59% identities with their human counterparts (Lagasse and Weissman, 1992), reflecting possible functional differences between the mouse and human proteins (Foell *et al.*, 2007).

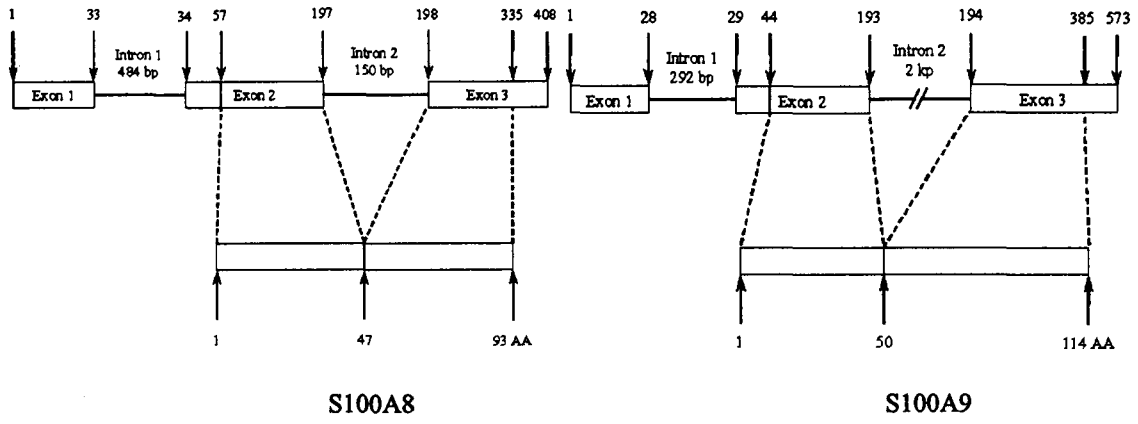

Fig. 1-3. Gene structure of human S100A8 and S100A9. *(Adapted from Kerkhoff et al., 1998)*

1.2.3 S100A8/S100A9 Complex Formation

Human S100A8 and S100A9 tend to form non-covalent heterodimers that appear to be the biologically active form within cells (Edgeworth *et al.*, 1991; Hunter and Chazin, 1998; Newton and Hogg, 1998; Propper *et al.*, 1999; Teigelkamp *et al.*, 1991). The human S100A8/S100A9 heterodimer has been shown to exhibit an enhanced complementarity of the interface compared to the homodimer forms of S100A8 and S100A9 (Hunter and Chazin, 1998). The two proteins can also form various hetero-oligomeric complexes, including functionally relevant heterotrimers (Andersson *et al.*, 1988; Siegenthaler *et al.*, 1997; Teigelkamp *et al.*, 1991) and Ca^{2+}-induced heterotetramers (Edgeworth *et al.*, 1991; Leukert *et al.*, 2006; Propper *et al.*, 1999; Strupat *et al.*, 2000; Teigelkamp *et al.*, 1991). The C-terminal EF-hand of S100A9 is suggested to facilitate the interactions between S100A8 and S100A9 and determine the specificity of dimerization (Propper *et al.*, 1999). High-resolution three-dimensional structures of human S100A8 and S100A9 proteins, as well as the heterodimer, have been published (Ishikawa *et al.*, 2000; Itou *et al.*, 2002; Korndorfer *et al.*, 2007).

It has also been suggested that mouse S100A8 exists primarily as a heterodimer with S100A9 (Sorg, 1992). However, in a study by Propper *et al.* (1999) of the interaction between S100A8 and S100A9 using a yeast two-hybrid system, it was demonstrated that the mouse S100A8 and S100A9 proteins readily form homodimers (although there was a stronger preference to form the heterodimeric complex), whereas homodimerization of the human proteins was not detected. This came as a surprise since the primary structure of the two proteins in both species is highly homologous (Propper *et al.*, 1999). Furthermore, in contrast to the coordinated expression of S100A8 and S100A9 in human myeloid cells (Roth *et al.*, 1993b), several authors have reported expression of the two mouse proteins independent of each other (Hsu *et al.*, 2005; Hu *et al.*, 1996; Passey *et al.*, 1999a; Rahimi *et al.*, 2005; Xu *et al.*, 2001; Xu and Geczy, 2000; Yen *et al.*, 1997), pointing towards structural and functional differences in the two species.

1.2.4 Distribution of S100A8 and S100A9

Expression of S100A8 and S100A9 is restricted to neutrophils and monocytes/macrophages (Edgeworth *et al.*, 1991; Hessian *et al.*, 1993; Roth *et al.*, 1992), as well as different types of epithelium during inflammatory states (Brandtzaeg *et al.*, 1987; Dale *et al.*, 1989; Kunz *et al.*, 1992; Wilkinson *et al.*, 1988). The S100A8/S100A9 heterodimer makes up a significant proportion (~45%) of the cytosolic proteins of circulating and interstitial neutrophils (Edgeworth *et al.*, 1991; Fagerhol and Anderson, 1980). The complex is also expressed to a lesser degree in monocytes (~1% of monocyte cytosolic proteins) in the early stages of differentiation and in infiltrating monocytes (Hessian *et al.*, 1993; Lagasse and Clerc, 1988), but not in resident (mature) tissue macrophages (Zwadlo *et al.*, 1988).

The tissue distribution of both proteins is similar in the murine and human systems (Zwaldo *et al.*, 1988). Murine S100A8 and S100A9 are also constitutively expressed in neutrophils and monocytes (Lagasse and Weissman, 1992), and like their human counterparts, their expression is associated with an intermediate level of macrophage differentiation (Goebeler *et al.*, 1993). This differential expression pattern is also observed for (and seems to depend on) different inflammatory conditions (Brun *et al.*, 1994; Hogg *et al.*, 1985, 1989; Odink *et al.*, 1987; Sorg, 1992; Zwaldo *et al.*, 1988).

1.2.5 Secretion of S100A8 and S100A9

S100A8 and S100A9 are secreted to the extracellular milieu by neutrophils and activated monocytes/macrophages (Dean *et al.*, 1997; Frosch *et al.*, 2000; Hetland *et al.*, 1998; Teigelkamp *et al.*, 1991), and their presence, therefore, is a marker for inflammatory cell accumulation (Foell *et al.*, 2004; Nacken *et al.*, 2003). Numerous studies have confirmed the release of the two proteins as a heterodimer from stimulated granulocytes and monocytes (Hetland *et al.*, 1998; Rammes *et al.*, 1997; Voganatsi *et al.*, 2001), as well as activated human keratinocytes (Thorey *et al.*, 2001) and phorbol 12-myristate 13-acetate (PMA)-treated monocytic THP-1 leukemia cells (Murao *et al.*, 1990). Murine S100A8 is released following neutrophil activation and phagocytosis *in vitro* and *in vivo* (Kumar *et al.*, 2001). Constitutive secretion of S100A8/S100A9 is also reportedly seen in the murine granulocytic 32D cell line (Nacken *et al.*, 2000).

Activated phagocytes secrete large amounts of S100A8/S100A9 upon contact with the endothelium at inflammatory sites (Eue *et al.*, 2000; Frosch *et al.*, 2000; Hogg *et al.*, 1989), which may explain the correlation of their concentration in serum and body fluids with disease activity in different acute and chronic inflammatory conditions,

including rheumatoid arthritis, and inflammatory bowel and lung disease (Brun *et al.*, 1994; Foell *et al.*, 2004; Foell and Roth, 2004; Kumar *et al.*, 2001; Odink *et al.*, 1987; Roth *et al.*, 1992, 2003; Zwaldo *et al.*, 1988).

The mechanism of S100A8/S100A9 release is not well understood since S100 proteins lack a conventional signal peptide sequence to mediate their release from cells (Donato, 2001, 2003). Studies have shown that the complex is secreted via a novel pathway that depends on an intact microtubule network and involves the activation of protein kinase C, bypassing the classical endoplasmic reticulum-Golgi route (Rammes *et al.*, 1997; Voganatsi *et al.*, 2001).

1.2.6 Functions of S100A8 and S100A9

Numerous functions for S100A8 and S100A9 have been proposed, although they have not been fully elucidated *in vivo*. The two proteins are emerging as important mediators in inflammation and cancer (Bhardwaj *et al.*, 1992; Foell *et al.*, 2007, Gebhardt *et al.*, 2006). Expression of S100A8 and S100A9 is up-regulated in mouse and human skin cancer cells (Gebhardt *et al.*, 2002) as well as human prostate cancer cells (Hermani *et al.*, 2006). This section will briefly outline intra- and extracellular functions of S100A8 and S100A9, with an emphasis on their role in modulating inflammatory processes.

1.2.6.1 Intracellular functions of S100A8 and S100A9

The S100A8/S100A9 complex, being the most abundant calcium-binding protein in neutrophils, is suggested to function in calcium homeostasis by regulating intracellular Ca^{2+} levels (Hessian *et al.*, 1993). S100A8 and S100A9 are found predominantly in the cytoplasm, but upon increases in intracellular Ca^{2+} levels, they are translocated to the

plasma membrane in activated human neutrophils and monocytes, where they interact with cytoskeletal filament proteins and, thus, may have roles in cell migration, phagocytosis and signal transduction (Bhardwaj *et al.*, 1992; Lemarchand *et al.*, 1992; Rammes *et al.*, 1997; Roth *et al.*, 1993a).

The complex may also play an important role in fatty acid metabolism, particularly of arachadonic acid (AA) in activated neutrophils by specifically binding AA in a Ca^{2+}-dependent manner, thereby regulating its mobilization, metabolization and release (Kerkhoff *et al.*, 1999; Klempt *et al.*, 1997). Since AA is a key inflammatory intermediate, the interactions between S100A8/S100A9 and AA may point towards a role for the complex in perpetuating the inflammatory response (Nacken *et al.*, 2003). The AA-binding capacity of S100A8/S100A9 is reversed by Zn^{2+} and Cu^{2+}, but unaffected by hypochlorite oxidation (Kerkhoff *et al.*, 1999).

Furthermore, S100A8/S100A9 has been shown to regulate the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a membrane-bound enzyme in phagocytes activated during respiratory burst (Doussiere *et al.*, 2002). By transferring electrons from NADPH to molecular oxygen, NADPH oxidase generates superoxide (O_2^-), which acts as a defense mechanism against invading pathogens or in cellular signaling (Doussiere *et al.*, 2002). This is possibly mediated by S100A8/S100A9 binding AA, an activator of NADPH oxidase (Doussiere *et al.*, 2002; Kerkhoff *et al.*, 2005). S100A8/S100A9 has also been shown to interact directly with cytochrome *b* (the redox centre of NADPH oxidase), altering its conformation to enhance the enzyme's activity (Berthier *et al.*, 2003).

S100A8 and S100A9 are also suggested to play a role in myeloid cell differentiation, as seen in their expression pattern, which is restricted to specific functional stages of these cells (Lagasse and Clerk, 1988; Roth *et al.*, 1993b; Teigelkamp *et al.*, 1991). The expression of S100A8 and S100A9 is inducible in human promyelocytic leukemia (HL-60) cells by differentiation along the monocytic lineage with 1α, 25-dihydroxy vitamin D3 and the granulocytic lineage with dimethyl sulfoxide (DMSO) (Collins *et al.*, 1979; Kuwayama *et al.*, 1993; Murao *et al.*, 1983; Warner-Bartnicki *et al.*, 1993). Moreover, induction of a mature macrophage phenotype with PMA causes S100A8 and S100A9 mRNA levels to disappear (Lagasse and Clerk 1988; Murao *et al.*, 1983; Roth *et al.*, 1993b), as seen in monocyte maturation *in vitro* (Zwadlo *et al.*, 1988).

S100A8/S100A9 may also regulate phosphorylation and enzyme activity through the inhibition of the multi-functional protein kinases, casein kinases I and II (Hessian *et al.*, 1993; Murao *et al.*, 1989). Casein kinase II is required for the phosphorylation of Myc transcription factors, RNA polymerase I and II, tumour-suppressor protein p53, as well as a range of other target proteins important for cell cycle control and cellular transcription and translation (Warner-Bartnicki *et al.*, 1993).

1.2.6.2 Extracellular functions of S100A8 and S100A9

In the extracellular environment, the S100A8/S100A9 complex has been suggested to exhibit various functions, including AA transport to target cells (Kerkhoff *et al.*, 2001), apoptosis (Viemann *et al.*, 2007), cytostatic and anti-microbial effects (Steinbakk *et al.*, 1990; Yui *et al.*, 1995), anti-oxidant effects (Harrison *et al.*, 1999; McCormick *et al.*, 2005), activation of the Toll-like receptor 4 (TLR4) (Vogl *et al.*,

2007), activation of endothelial cells (Viemann *et al.*, 2005) and chemotactic activities for neutrophils and monocytes (Devery *et al.*, 194; Hobbs *et al.*, 2003; Lackmann *et al.*, 1992, 1993; Ryckman *et al.*, 2003b; Vogl *et al.*, 2004). S100A8 and S100A9 were recently described as damage-associated molecular patterns (DAMPs), which, upon release into the extracellular environment, signal tissue damage to the innate immune system (Foell *et al.*, 2007). The following sections will review the chemotactic and pro-inflammatory properties of the two proteins.

Chemotactic activities of murine S100A8

Chemotactic activity for neutrophils and monocytes was first attributed to murine S100A8, which induced sustained leukocyte recruitment *in vivo* as a homodimer or as a complex with S100A9 (Cornish *et al.*, 1996; Devery *et al.*, 1994; Geczy, 1996; Lackmann *et al.*, 1992, 1993; Passey *et al.*, 1999b; Ryckman *et al.*, 2003b). It is proposed that murine S100A8 alters the shape and size of neutrophils and monocytes, promoting their retention in microcapillaries and the onset of inflammation (Cornish *et al.*, 1996).

The chemotactic function of murine S100A8 is ascribed to the hinge domain (residues 42-55) of the protein (Lackmann *et al.*, 1993). Recently, it was demonstrated that S100A8 mediates pro-inflammatory functions via TLR4 (Vogl *et al.*, 2007), which has also been shown to regulate induction of S100A8 by LPS in human and murine macrophages (Hsu *et al.*, 2005; Xu *et al.*, 2001; Xu and Geczy, 2000). Vandal *et al.* (2003) showed that passive immunization of mice with specific anti-A8 and anti-A9 antibodies diminished LPS-induced neutrophil migration in air pouch studies. The authors also demonstrated that S100A8/S100A9 induced mobilization of neutrophils from the bone marrow into circulation (Vandal *et al.*, 2003). These findings indicate that

the two proteins are involved in neutrophil migration and sequestration to inflammatory sites, with an important role in mediating inflammatory responses (Kerkhoff *et al.*, 2005).

Chemotactic activities of human S100A8 and S100A9

Studies regarding the chemotactic properties of human S100A8 and S100A9 are controversial. The study by Ryckman *et al.* (2003b) demonstrated that human S100A8, S100A9 and S100A8/S100A9 induce neutrophil chemotaxis and adhesion to fibrinogen. Purified recombinant human S100A8, S100A9, or S100A8/S100A9 injected into a murine air pouch caused accumulation of neutrophils, verifying the chemotactic activity of the human proteins *in vivo* (Ryckman *et al.*, 2003a/b). Other reports, however, have provided conflicting results regarding the role of human S100A8 and S100A9 in monocyte and neutrophil adhesion and chemotaxis. In contrast to its murine counterpart, human S100A8 was suggested to lack chemotactic ability for neutrophils (Newton and Hogg, 1998). Human S100A9 was shown to stimulate neutrophil adhesion to fibrinogen without causing changes in cell shape, actin polymerization, or neutrophil migration (Newton and Hogg, 1998). This adhesion was reversed upon heterodimer formation with S100A8, indicating that S100A8 and the heterodimer do not induce neutrophil adhesion (Newton and Hogg, 1998). Eue *et al.* (2000), however, demonstrated that S100A9 and S100A8/S100A9, but not S100A8, induced monocyte adhesion to the endothelium. Further studies are required to substantiate the roles of human S100A8 and S100A9 in the recruitment of neutrophils and their transmigration into tissues at inflammatory sites.

The differences in the chemotactic ability of S100A8 in humans and mice may reflect distinct functions in the two species. Interestingly, human S100A12, a functional ortholog of murine S100A8 that is not expressed in mice, exhibits strong chemotactic

activity for neutrophils and monocytes and may have evolved to augment the relatively weak chemotactic activity of human S100A8 (Rouleau *et al.*, 2003; Yang *et al.*, 2001).

S100A8 as an oxidant scavenger

S100A8 exhibits a strong oxidant-scavenging capacity, particularly for hypochlorite and H_2O_2 (Harrison *et al.*, 1999; McCormick *et al.*, 2005). Oxidation of the single Cys-41 residue of S100A8 by hypochlorite, a major oxidant generated by activated phagocytes, has been shown to negatively regulate the chemotactic ability of murine S100A8 (Harrison *et al.*, 1999). In this study, hypochlorite converted 70-80% of the monomeric and chemotactic S100A8 to the disulfide-linked homodimer form, which was chemotactically inactive (Harrison *et al.*, 1999). Human S100A8 also fails to maintain its chemotactic capacity for neutrophils upon strong hypochlorite oxidation (Ryckman *et al.*, 2003b). This lost ability of S100A8 to recruit neutrophils and monocytes *in vivo* upon oxidation may be important for limiting excess accumulation of inflammatory cells and shielding other tissue proteins from oxidative damage (Harrison *et al.*, 1999). Interestingly, S100A9 does not form non-covalent homodimers under oxidizing conditions (Harrison *et al.*, 1999), emphasizing differences in the properties and/or functions of individual S100A8 and S100A9 dimers (Donato, 2003).

1.3 CYSTIC FIBROSIS

Cystic fibrosis (CF) is an autosomal recessive genetic disorder, the most common fatal inherited disease among Caucasian populations, with a frequency of approximately one in 2,500 live births (Riordan, 1993; Welsh and Smith, 1995). It is a disease of the exocrine glands affecting many tissues and organ systems, leading to a wide range of pathological and clinical problems (Pilewski and Frizzell, 1999; Welsh and Smith, 1995).

1.3.1 Clinical Manifestations of CF

In CF, nearly all exocrine tissues show some abnormality in ion and fluid transport, including the respiratory tract, pancreas, liver, gastrointestinal tract, vas deferens and sweat glands (Collins, 1992; Pilewski and Frizzell, 1999; Welsh and Smith, 1993). Patients with CF are unable to transport chloride properly through their epithelial cell membranes; consequently, fluid secretion into the luminal compartment is reduced, leading to a thick, stagnate mucus layer (Quinton, 1983; Riordan, 1993). Clinical manifestations of CF include obstruction of the airways and chronic respiratory infections, bile duct obstruction, intestinal obstruction, pancreatic insufficiency, infertility in males, chronic sinusitis and elevated electrolytes in the sweat (Collins, 1992; Welsh and Smith, 1993, 1995).

In the lung, the highly viscous mucus confers much more severe consequences, with recurrent bacterial infections and severe inflammation characterized by recruitment and infiltration of neutrophils, leading to progressive tissue deterioration (Riordan, 1993). The leading cause of death for more than 90% of CF patients who survive the neonatal period is respiratory failure due to pulmonary disease (Pilewski and Frizzell, 1999; Riordan, 1993). Although the basic biochemical defect in CF, a dysfunctional chloride

channel, was discovered in 1989 (Riordan *et al.*, 1989), many questions remain unanswered about how this defect leads to progressive inflammatory lung disease.

1.3.2 The CFTR Gene

CF results from mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein channel (Riordan *et al.*, 1989; Rommens *et al.*, 1989). The CFTR gene, located at region q31.2 on human chromosome 7, consists of 27 exons spread over 250,000 bp (Riordan *et al.*, 1989; Rommens *et al.*, 1989).

1.3.3 CFTR Protein Structure

The CFTR gene encodes a 1480 amino acid protein, a member of the ATP binding cassette (ABC) transport protein superfamily (Riordan *et al.*, 1989). The protein is composed of five domains: two transmembrane regions (TMD1 and TMD2), each consisting of six α-helices that form the pore of the ion channel; two nucleotide binding domains (NBD1 and NBD2) that bind and hydrolyze ATP; and a cytoplasmic regulatory (R) domain between the NBDs required for channel activation upon cyclic AMP (cAMP)-dependent protein kinase A (PKA) phosphorylation (Sheppard and Welsh, 1999) (Fig. 1-4). The two glycosylated side chains on the extracellular loop between transmembrane domains seven and eight are added to the mature protein during processing (Sheppard and Welsh, 1999).

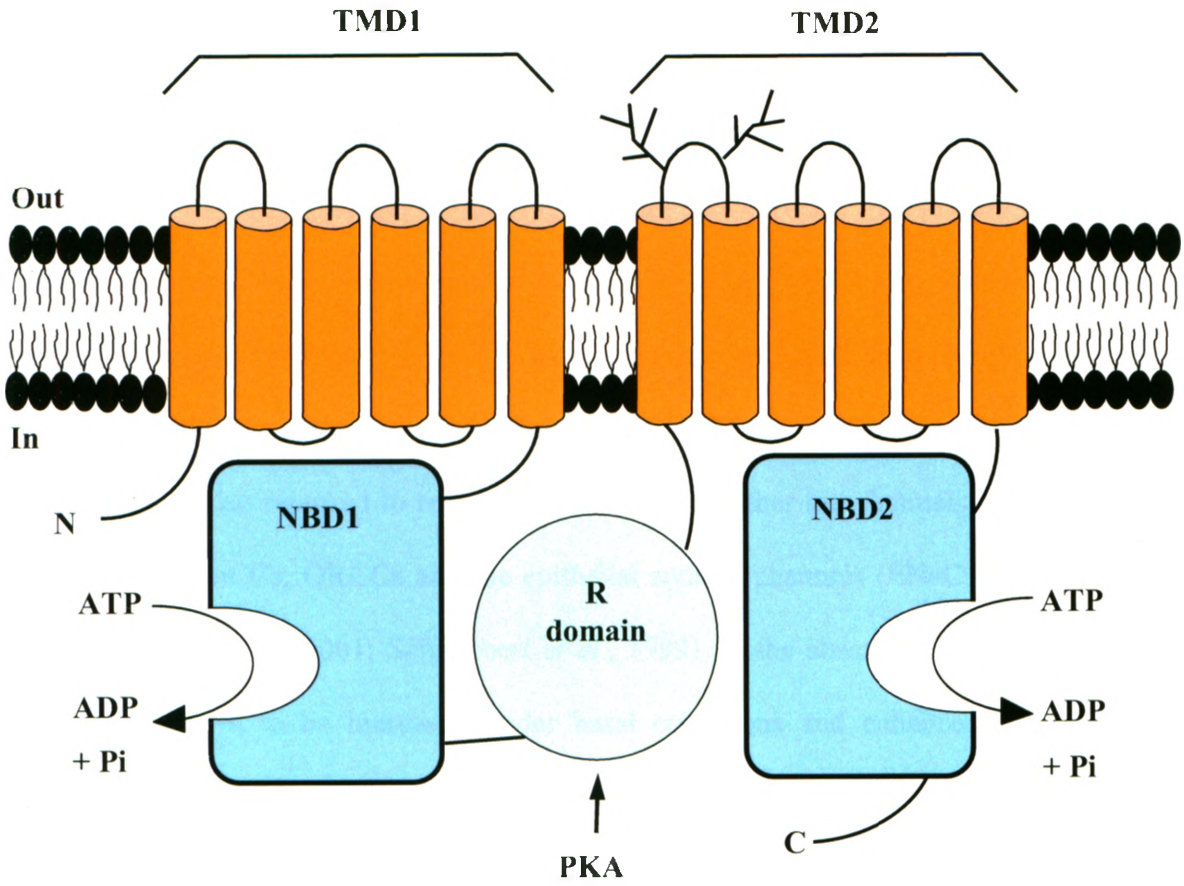

Fig. 1-4. The proposed structure of CFTR. A schematic representation of the CFTR protein and its five domains: two transmembrane regions (TMDs) each consisting of six transmembrane alpha helices; two ATP-hydrolyzing nucleotide binding domains (NBDs); and a regulatory (R) domain between the NBDs. Phosphorylation of the R domain by cAMP-dependent PKA activates the channel. *(Adapted from Sheppard and Welsh, 1999)*

1.3.4 Functions of CFTR

CFTR is a cAMP-dependent chloride channel in the apical membrane of epithelial cells that transports Cl^- out of cells and into the luminal compartment (Pilewski and Frizzell, 1999; Riordan, 1993; Row *et al.*, 2005). Other ion channels may also contribute to Cl^- secretion, including the Ca^{2+}-activated chloride channels (CaCCs), the outwardly rectifying chloride channels (ORCCs), and the voltage-sensitive chloride channels (ClCs) (Pilewski and Frizzell, 1999; Quinton, 1983). This Cl^- secretion generates a negative potential that draws Na^+ and fluid into the lumen (Fig. 1-5A). CFTR also transports other anions, including bicarbonate (HCO_3^-) in pancreatic duct epithelial cells (Ishiguro *et al.*, 2009), with a selective pattern of $Cl^- > I^- > Br^- > NO_3^- > HCO_3^-$ (Linsdell *et al.*, 1997).

CFTR is also reported to regulate the function of other ion channels in epithelial cells, including CaCCs, ORCCs and the epithelial sodium channels (ENaCs) (Greger *et al.*, 1996; Kunzelmann, 2001; Schwiebert *et al.*, 1999). In the absence of CFTR, ENaC activity was shown to be increased under basal conditions and enhanced further by cAMP, whereas in the presence of functional CFTR, ENaC activity was reduced and the effect of cAMP became inhibitory (Stutts *et al.*, 1995). The mechanisms of this interaction, however, remain elusive. Moreover, since the CFTR protein is anchored in close proximity to a number of receptors and the cytoskeleton, it may also interfere with the expression of other proteins, including those involved in signaling pathways of the inflammatory response (Sheppard and Welsh, 1999).

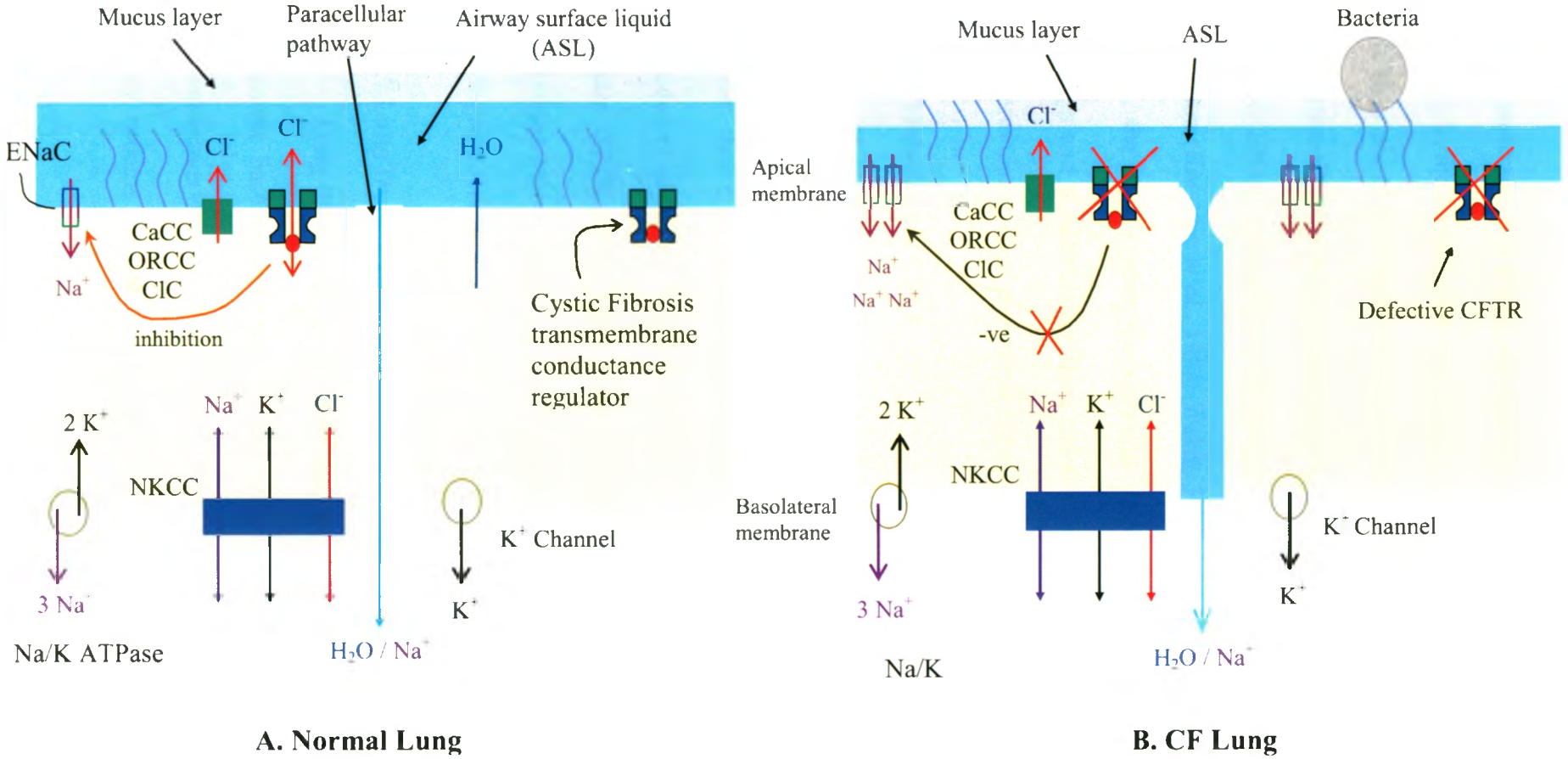

Fig. 1-5. Comparison of models of basic ion transport in normal and CF airway epithelia. (A) Cl^- is transported across the apical membrane into the luminal compartment by CFTR, Ca^{2+}-activated chloride channels (CaCCs), outwardly rectifying chloride channels (ORCCs), and voltage-sensitive chloride channels (ClCs), and across the basolateral membrane by the $Na^+/K^+/2Cl^-$ co-transporter (NKCC). Na^+ follows into the lumen through the paracellular pathway. This generates a driving force for fluid secretion into the luminal compartment of the airways. **(B)** When CFTR is mutated, Cl^- secretion is diminished, and this, combined with the hyperactivity of epithelial sodium channels (ENaCs) which facilitate Na^+ absorption into the cells, creates an abnormally high osmotic drive for fluid re-absorption.

1.3.5 Defective Ion Transport in CF Airways

In the lung, CFTR is expressed in surface epithelial cells and submucosal glands (Chu *et al.*, 1991; Engelhardt *et al.*, 1994). The lack of CFTR function leads to complete or partial absence of Cl^- secretion and this, combined with the excessive absorption of Na^+ *via* ENaCs, produces an abnormally high osmotic driving force for the re-absorption of water (Knowles *et al.*, 1983; Welsh, 1987; Welsh and Smith, 1993) (Fig 1-5B). As a result, the mucus lining the epithelial surfaces becomes viscous, which obstructs the airways and impairs mucocillary action (Collins *et al.*, 1992; Welsh and Smith, 1993). This provides an ideal environment for bacterial invasion and growth.

1.3.6 Mutations of CFTR

The most common mutation in the CFTR gene is ΔF508, a deletion of a phenylalanine residue at the 508th amino acid position, which produces a misfolded protein that is retained in the ER, rendering it non-functional (Kerem *et al.*, 1989; Riordan *et al.*, 1989). The ΔF508 mutation is present in approximately 70% percent of mutant CFTR alleles (Rowe *et al.*, 2005). There are more than 1500 other known mutations that result in CF (Cystic Fibrosis Genetic Analysis Consortium, www.genet.sickkids.on.ca/cftr/)

1.3.7 Lung Disease and Inflammation

Lung disease in CF is characterized by a heightened inflammatory response to persistent bacterial infection (Welsh and Smith, 1995). *Staphylococcus aureus* and *Pseudomonas aeruginosa* are common sources of infection in the lungs of CF patients, which become trapped in the thick mucus build-up in the bronchioles (Pilewski and

Frizzell, 1999). Obstruction of the airways is attributed to a number of factors, including mucus accumulation, goblet cell hyperplasia, submucosal gland hypertrophy, tissue inflammation and structural damage of the lung parenchyma and subsequent loss of elastic recoil (Lundgren and Vestbo, 1995).

The hallmark of CF lung disease, an early and inappropriate influx of neutrophils and dysregulation in the levels of inflammatory cytokines, is suggested to contribute to the vicious cycle of inflammation and infection, leading to tissue destruction and respiratory failure (Bonfield *et al.*, 1995; Kammouni *et al.*, 1997; Konstan and Berger, 1997). Interestingly, young CF patients present with a heightened inflammatory response in the absence of infection (Armstrong *et al.*, 1997; Balough *et al.*, 1995; Muhlebach *et al.*, 1999; Rosenfeld *et al.*, 2001), so it is not clear whether the inflammation precedes the infection or *vice versa*. The failure to control the inflammatory response, therefore, appears to be an inherent feature of CF. This section will highlight some aspects CF lung disease, with an emphasis on the role of neutrophils in the persistent inflammation.

1.3.7.1 Mucus Abnormalities

The airway surface liquid (ASL) covering the apical surface of the airway epithelium acts as the first line of defense against pathogens and is required for proper mucociliary clearance (Sade *et al.*, 1970). The mucus layer of the ASL contains anti-microbials, such as lactoferrin, lysozyme and defensins, which can inhibit bacterial growth (Terheggen-Lagro *et al.*, 2005). The amount and composition of the ASL are controlled by ion transport across the epithelial cells of the airway (Terheggen-Lagro *et al.*, 2005). Loss of CFTR function causes increased salt and water absorption, which leads to the depletion of the ASL layer and consecutive breakdown of mucociliary

transport (Sade *et al.*, 1970). Increased mucus production by submucosal glands and goblet cells and decreased mucociliary clearance contribute to the increased mucus volume in the lumen of the airways (Lundgren and Vestbo, 1995). Mucus production may also be stimulated by inflammatory mediators generated by neutrophils and monocytes/macrophages (Lundgren and Vestbo, 1995). The antimicrobials within these tethered mucus plaques eventually become ineffective, promoting the growth of microbes (Terheggen-Lagro *et al.*, 2005).

1.3.7.2 Cytokine Dysregulation

Alterations in the levels of inflammatory mediators in CF airway epithelial cells may contribute to the hyper-inflammatory response observed in CF lungs. Pro-inflammatory cytokines such as the interleukins IL-1β, IL-6 and IL-8, and tumor necrosis factor-alpha (TNF-α), have been shown to be elevated in CF epithelial cells (Bonfield *et al.*, 1995, 1999; Kammouni *et al.*, 1997; Pfeffer *et al.*, 1993) as well as airway and blood neutrophils (Corvol *et al.*, 2003), whereas levels of the anti-inflammatory cytokine IL-10 are decreased (Bonfield *et al.*, 1995). IL-10 has been shown to down-regulate the secretion of S100A8/S100A9 by stimulated monocytes *in vitro* in a dose-dependent manner (Lugering *et al.*, 1997). Increased production of TNF-α could directly induce inflammation in CF lungs by activating a cascade of other inflammatory mediators that enhance neutrophil recruitment and activation (She *et al.*, 1989). The production of these cytokines is regulated by nuclear factor-κB (NF-κB) (Barnes *et al.*, 1997; Blackwell *et al.*, 2001), a transcription factor shown to be highly active in CF epithelial cells (Weber *et al.*, 2001). These findings are consistent with dysregulated cytokine production and enhanced pro-inflammatory signaling in CF cells.

1.3.7.3 Neutrophil Recruitment

Neutrophils play an important role in innate defense against infections by containing pathogenic populations through phagocytosis as well as the release of toxic substances such as pro-inflammatory cytokines, proteases and reactive oxygen species (Hammerle *et al.*, 1997). This manifests as an inflammatory phenotype, which, in healthy lungs, subsides naturally and the epithelial tissue returns to normal (Henson, 2003). In CF, however, the inflammatory response is severe, sustained by a cycle of neutrophil recruitment and infiltration into the tissues of the airways (Konstan and Berger, 1997; Pilewski and Frizzell, 1999). Transendothelial migration occurs along a chemotactic gradient, whereby neutrophils penetrate the endothelial layer and migrate towards the site of inflammation (Ley, 1996).

Neutrophils of CF patients are less sensitive to apoptosis (McKeon *et al.*, 2003) and display a heightened (pre-activation) 'primed' state (Terheggen-Lagro *et al.*, 2003). The build-up of proteases and activated oxygen radicals (particularly H_2O_2 and O_2^-) released from accumulated neutrophils leads to severe pulmonary damage (Konstan and Berger, 1997; McElvaney and Crystal, 1999). CF neutrophils are also triggered by TNF-α and IL-8 to release large amounts of elastase (Tagart *et al.*, 2000), which directly damages lung tissue by degrading structural proteins such as elastin, collagen and glycoproteins (McElvaney and Crystal, 1999).

These findings are suggestive of a defect in the processes responsible for the resolution of inflammation and restoration of tissue homeostasis, normally achieved through apoptosis of neutrophils followed by their clearance and release of anti-inflammatory mediators (Haslett, 1999).

1.4 GENETIC MODIFIERS OF CF

There is evidence to suggest that CFTR may have functions that extend well beyond simple chloride conductance in epithelial cells. In humans with CF as well as CFTR-knockout mice, the phenotypic consequences of the absence of the CFTR gene product appear to be influenced by the expression of other 'modifier' genes, including genes important in ion transport, cell signaling and inflammation (Rowe *et al.*, 2005).

To identify relevant factors that may underlie these genetic modifiers, we have previously undertaken a comprehensive study of differential gene expression among 3-week old congenic C57BL/6 CF (B6-CF) and BALB/c CF (Bc-CF) and wild-type mouse lungs using Affymetrix GeneChip® technology. The GeneChip® experiments were performed using stringent criteria for identifying genes that could contribute, through differential expression, to the distinct CF-associated lung phenotypes among the different mice. Briefly, total RNA from congenic B6-CF lung samples (2 male and 2 female) were hybridized to GeneChips® (spotted with sequences of over 18,000 known or potential genes) along with RNA from their wild-type (sex-matched) siblings. Likewise, identical hybridization experiments were carried out with congenic Bc lung samples. The signals of all eight independent hybridization experiments were quantified and spots showing a difference in expression in excess of 2-fold that were consistent among the four hybridization experiments for each strain were considered significant. The significant signals between the B6 and Bc strains were then compared and only those that were different between the two strains were considered for further investigation as potential contributors to distinct lung disease phenotypes. Of the 31 (from >18,000) most promising genes meeting the stringent criteria, and confirmed to be differentially

expressed by Northern blot or quantitative RT-PCR analysis, a large proportion corresponded to inflammatory mediators and structural proteins.

The studies conducted by our lab, as well as others, have identified S100A8 and S100A9 as potential modifiers of the inflammatory lung phenotype in CF (Andersson *et al.*, 1988; Haston *et al.*, 2002; Tirkos *et al.*, 2001, 2006). Another modifier gene, the calcium-activated chloride channel 3 (CLCA3), has been shown to influence intestinal disease in CF mice, with functions related to mucus production and composition (Young *et al.*, manuscript in preparation). The identification and characterization of modifier genes for CF will help to explain the substantial variability in clinical severity among patients with the same mutations in the CFTR gene, and may offer ways to find novel therapies for CF (Rowe *et al.*, 2005).

1.5 ROLE OF S100A8 AND S100A9 IN CF LUNG DISEASE

Neutrophils and monocytes are the main producers of S100A8/S100A9 (Hessian *et al.*, 1999). Since the concentration of S100A8/S100A9 is abnormally elevated in the lungs and serum of CF patients, it has been postulated that this factor has important functions influencing the inflammatory processes in CF (Andersson *et al.*, 1988; Barthe *et al.*, 1991; Sorg, 1992; Wilkinson *et al.*, 1988).

In acute inflammatory states, as circulating monocytes are recruited into tissues, they differentiate, and in the process show different levels of expression of S100A8 and S100A9 (Zwaldo *et al.*, 1988). As neutrophils and monocytes migrate deeper into the site of inflammation, the expression of the two proteins is normally turned off (Roth *et al.*, 1992). Lungs of mice infected with the influenza virus showed S100A8 expression, which declined at the beginning of the recovery phase, indicating that, under normal

circumstances, S100A8 gene expression is down-regulated by mediators acting at the resolution phase of inflammation (Endoh *et al.*, 2009). In contrast, in some chronic inflammatory conditions, expression of S100A8 and S100A9 is sustained in neutrohils and tissue macrophages, suggesting that there is a regulatory defect in these cells in chronic inflammatory states (Hessian *et al.*, 1993; Tirkos *et al.*, 2006). Interestingly, the S100A8/S100A9 protein complex was shown to be highly resistant to proteolysis in the presence of calcium (Nacken *et al.*, 2007), which may account for the protein's stability and function at inflammatory sites in the presence of high concentrations of proteases.

Thus, the failure to down-regulate S100A8 and S100A9 expression by neutrophils and tissue macrophages upon entering the lung interstitium, as well as the increased levels of neutrophil activators, such as IL-6, IL-8 and TNF-α, may explain the massive neutrophil recruitment and inflammatory phenotype observed in CF lungs.

1.6 MOUSE MODELS OF CF

Mouse models have been an important tool for the study of CF. The murine CFTR gene contains 27 exons spanning approximately 152,000 bp on chromosome 6 (Tata *et al.*, 1991). The generation of the first CF mouse model (*Cftr*^{tm1UNC}) was reported in 1992, in which the CFTR gene was disrupted by exon 10 replacement using gene targeting resulting in defective CFTR production (Snouwaert *et al.*, 1992). Since then, a number of other mouse models of CF have been created (Delaney *et al.*, 1996; Ratcliff *et al.*, 1993; Rozmahel *et al.*, 1996; Thomas *et al.*, 2000) using a variety of methods (Davidson and Rolfe, 2001). Of particular interest to us are mouse models that develop an inflammatory lung phenotype as observed clinically in patients with CF. The original CFTR-knockout mouse did not develop lung disease, despite the presence of severe

intestinal disease (Snouwaert *et al.*, 1992). In 1997, Kent *et al.* produced a congenic CFTR-knockout mouse that acquired spontaneous and progressive lung disease, characterized by failure of effective mucociliary clearance, acinar and alveoli hyperinflation, interstitial thickening, fibrosis and inflammatory cell recruitment, features consistent with obstructive small airway disease in CF patients (Oppenheimer *et al.*, 1975). The inflammatory phenotype was 'spontaneous' in that the mice were maintained in specific pathogen-free conditions. The authors suggested that the variability in the development of lung disease between the original mixed genetic background and congenic CFTR-knockout strains may be due, in part, to the presence of alternative Cl^- channels in the original model that could compensate for defective CFTR function (Kent *et al.*, 1997).

1.6.1 Targeted Knockdown of S100A8 and S100A9

Mouse models in which S100A8 and S100A9 expression has been altered are important in elucidating the role of these two proteins in the pathological features of CF lung disease. Hobbs *et al.* (2003) and Manitz *et al.* (2003) independently produced S100A9 null mice, which displayed a normal phenotype but their neutrophils and macrophages were less sensitive to chemo-attractants compared to their wild-type counterpart. In contrast, S100A8 null mouse embryos do not survive and are resorbed by the mother early in development (Passey *et al.*, 1999a). These S100A8 null embryos were infiltrated with maternal cells prior to resorption, suggesting a role for S100A8 in preventing maternal rejection of the implanting embryo (Passey *et al.*, 1999a).

1.6.2 Differential Expression of S100A8 and S100A8 in CF Mouse Models

Previously, our lab showed that congenic B6-CF mice exhibited a lung-specific inflammatory phenotype, characterized by an early (3-weeks of age) and spontaneous 3-fold elevation of S100A8 and S100A9 levels, compared to wild-type mice (Tirkos *et al.*, 2001, 2006). In addition, a significant increase in neutrophils was observed in B6-CF lungs at 4-weeks of age, corresponding to the elevated expression of S100A8 and S100A9. Importantly, congenic Bc-CF mice, which do not develop a lung inflammatory phenotype, showed elevated levels of S100A9 but not S100A8, suggesting that S100A8 exclusively (or in combination with S100A9) plays a prominent role in the early and spontaneous lung inflammation seen in B6-CF mice (Tirkos *et al.*, 2006).

1.6.3 S100A8 Transgenic Mouse Models

Using an S100A8 transgenic mouse model (Tg-S100A8), our lab showed that over-expression of S100A8 on a B6 background results in a lung-specific inflammatory phenotype that is indistinguishable from that of B6-CF mice at 3-weeks of age, with multifocal areas of interstitial and alveolar septa thickening, fibrous tissue deposition and inflammatory cell infiltration (Newbigging *et al.*, manuscript submitted) (Fig. 1-6A). Both B6-CF and Tg-S100A8 lungs showed a further and more profound interstitial thickening with a marked increase in collagen/fibrous tissue deposition at 6-months of age compared to wild-type lungs (Fig. 1-6B). It is important to note that the upsurge of S100A8 and S100A9 expression in CF lungs occurs just before the onset of inflammation (Tirkos *et al.*, 2006). This suggests that, rather than being a mere consequence of lung disease, the increase in S100A8 (or S100A8/S100A9) may underlie or exacerbate the inflammatory lung phenotype in CF mice (Newbigging *et al.*, manuscript submitted).

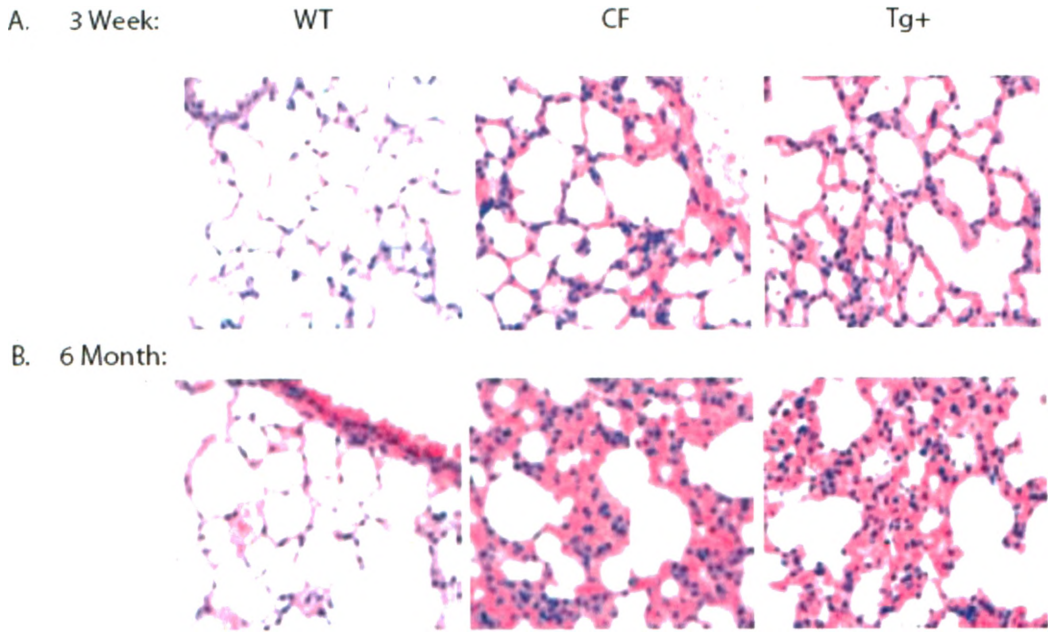

Fig. 1-6. Histopathological analysis of 3-week and 6-month old B6-CF and Tg-S100A8 lungs. (**A**) H&E stained 3-week old B6-CF and Tg-S100A8 (Tg+) lungs appear morphologically indistinguishable, with interstitial and alveolar septa thickening and inflammatory cell infiltration. **(B)** Both B6-CF and Tg-S100A8 lungs show a more profound phenotype at 6-months of age compared to the wild-type (WT) lungs. (Magnification: x40) (*Obtained from Newbigging et al., manuscript submitted)*

1.7 RNA INTERFERENCE

1.7.1 History of RNA Interference

RNA interference (RNAi) is a naturally occurring process in which short interfering RNA suppress gene expression via enzymatic cleavage and destruction of target mRNA (Rana, 2007). RNAi was first recognized in 1990 as a natural anti-viral biological response in plants (Napoli *et al.*, 1990). Long double-stranded RNA (dsRNA) produced during viral infections induces this natural mechanism, in which the dsRNA molecules are cleaved into smaller, 21-25 nt RNA duplexes called small interfering RNAs (siRNAs), which then direct sequence-specific gene silencing in the host cell (Waterhouse *et al.*, 2001).

Fire *et al.* (1998) first described the phenomenon by injecting dsRNA into the worm *Caenorhabditis elegans* which led to efficient suppression of target gene expression. Unlike the natural and target-specific RNAi response demonstrated in plants (Smith *et al.*, 2000), worms (Tavernarakis *et al.*, 2000) and fruit flies (Kennerdell and Carthew, 2000), introduction of exogenous long dsRNA (>30 bp) induces an anti-viral interferon response in mammalian cells (Sledz *et al.*, 2003). This response is characterized by non-specific inhibition of protein synthesis via a dsRNA-activated protein kinase R (PKR), which results in global suppression of translation, RNA degradation and apoptosis (Gil and Esteban, 2000; Williams, 1997). It was then revealed that this anti-viral response can be avoided by the introduction of short (21-25 nt) synthetic siRNA molecules that can be processed within cells by the natural RNAi machinery (Elbashir *et al.*, 2001a), making RNAi-mediated gene silencing a very useful and promising tool for studying gene function.

1.7.2 Triggers of RNAi

1.7.2.1 MicroRNAs

MicroRNAs (miRNAs) are genomically-encoded, untranslated 21-23 nt RNA molecules, which regulate diverse cellular processes, including cell proliferation, development, differentiation, apoptosis and metabolism by triggering the RNAi pathway (Bartel, 2004; Chen *et al.*, 2004; Cheng *et al.*, 2005; Xu *et al.*, 2004). The first miRNA identified in *C. elegans,* lin-4, showed incomplete base-pairing to complementary sequences on target mRNAs and blocked gene expression (Lee *et al.*, 1993). It was then discovered that mammalian genomes encoded hundreds of these miRNA genes which play an important role in regulating health and disease states (Bartel, 2004).

1.7.2.2 Small interfering RNAs

Small interfering RNAs (siRNAs) represent another class of regulatory 21-25 nt dsRNA molecules that can silence gene expression *via* the RNAi pathway. The discovery of siRNAs was made by Hamilton and Baulcombe (1999) as part of post-transcriptional gene silencing in plants. These siRNA duplexes are produced naturally when an RNAse-III type enzyme, Dicer, cleaves long dsRNA molecules (Rana, 2007). Alternatively, chemically synthesized siRNAs may be introduced into cells to induce RNAi. Elbashir *et al.* (2001a) first made the phenomenal discovery that synthetic 21 nt siRNAs transfected into mammalian cells can effectively inhibit gene expression in a sequence specific manner (Elbashir *et al.*, 2001a; Harborth *et al.*, 2001). However, there are some challenges as this technology transitions towards *in vivo* applications; namely, tissue specific delivery, low transfection efficiency and the transient nature of this method, which makes siRNAs unsuitable for long-term studies (Downward, 2004).

1.7.2.3 Short-hairpin RNAs

Some of the difficulties encountered with synthetic siRNAs have been addressed with alternative vector-based short-hairpin RNA (shRNA) expression systems, whereby siRNAs are generated continuously through their expression from plasmid or viral vectors (Brummelkamp *et al.*, 2002a/b; Paddison *et al.*, 2002; Sui *et al.*, 2002). In this case, the shRNA molecule consists of complementary strands of 21-29 nt spaced by a non-complementary short 'loop' region (that causes the transcript to fold back on itself when transcribed *in vivo*) and a short terminator sequence (Manjunath *et al.*, 2009) (Fig. 1-7). Recognition and processing of the shRNA transcripts by the RNAi machinery in the cytoplasm generates the active siRNAs that confer silencing (Wadhwa *et al.*, 2004).

For the expression of the shRNA, RNA polymerase III promoters, such as the human H1 or U6 snRNA promoters, are generally used to produce a large amount of transcripts that are processed by Dicer directly (Manjunath *et al.*, 2009). Short-hairpin RNAs have been shown to achieve effective (and stable) gene silencing in plants (Smith *et al.*, 2000), *C. elegans* (Tavernarakis *et al.*, 2000) and *Drosophila* (Kennerdell and Carthew, 2000). The application of shRNAs to induce sequence-specific silencing has also been demonstrated in mammalian cells (Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002), as well as *in vivo* (Liu *et al.*, 1999). Viral-based shRNA expression vectors, such as lentiviruses and retroviruses, have also been developed and shown to be effective in achieving knockdown *in vivo* (Carmell *et al.*, 2003; Gurzov *et al.*, 2006; Jiang *et al.*, 2006; Kunath *et al.*, 2003; Tiscornia *et al.*, 2003).

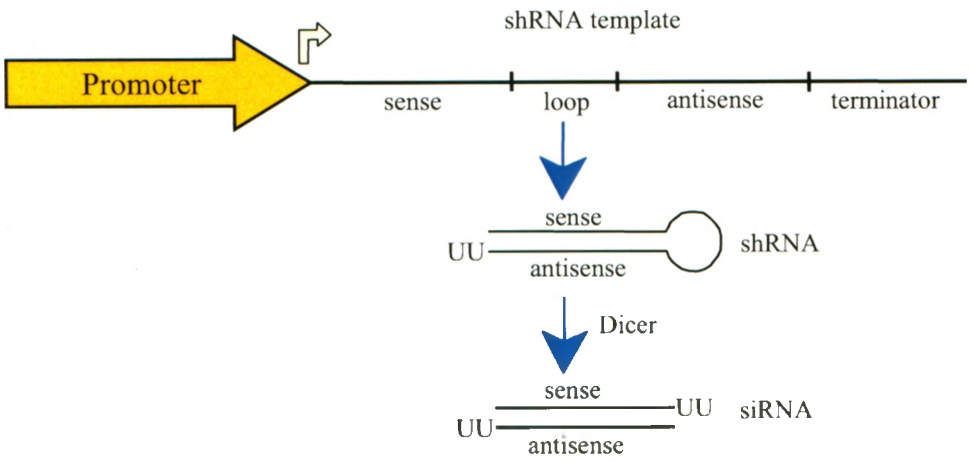

Fig. 1-7. Loop structure of shRNAs. After being transcribed from an expression vector, shRNA transcripts fold back onto themselves forming a 'hairpin' structure, consisting of two target-complementary 21-29 bp RNA sequences linked by a short loop of 4–10 nt. Dicer processing generates the active siRNAs that confer gene silencing.

1.7.3 Mechanism of RNAi

The function of siRNAs and shRNAs is mediated by the natural miRNA biogenesis pathway (Fig. 1-8). In this pathway, miRNAs are first transcribed by RNA polymerase II as long self-complementary dsRNA with a poly(A) tail and 5' cap, known as primary microRNA (pri-miRNA) (Lee *et al.*, 2002; Winter *et al.*, 2009). This transcript folds onto itself to form a stem-loop structure, with internal bulges resulting from imperfect homology between the two strands (Bartel, 2004; Winter *et al.*, 2009). Drosha, a special nuclear RNase III, processes this molecule into a 60-70 nt precursor miRNA (pre-miRNA), which is exported to the cytoplasm by the nucleocytoplasmic transporter exportin-5 (Yi *et al.*, 2003). In the cytoplasm, the ribonuclease Dicer, cleaves the pre-miRNA into 21-23 nt mature miRNA that assemble into the RNA-induced silencing complex (RISC) (Bartel, 2004; Winter *et al.*, 2009).

Usually only one strand (known as the guide strand) of the miRNA duplex is loaded into RISC, while the other strand (the passenger strand) is destroyed (Matranga *et al.*, 2005). The selection of the strand is governed by the relative thermodynamic stability of the 5' terminus of the miRNA molecule (Khvorova *et al.*, 2003). Studies have shown that the RISC complex is asymmetric and favors the strand of the RNA duplex with the least thermodynamically stable 5' end (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003). The miRNA-RISC complex recognizes and binds target mRNA leading to translational repression or mRNA degradation depending on the degree of complementarity between the two RNA molecules (Bernstein *et al.*, 2001; Lee *et al.*, 2004).

Endogenously encoded shRNAs are processed in a similar way as miRNAs (Fig. 1-9); however, shRNA transcripts are not cleaved by the nuclear Drosha (Rana, 2007).

Following their transcription and transport to the cytoplasm by exportin-5, the hairpins are processed into 21-23 nt double-stranded siRNAs by Dicer (Hammond *et al.*, 2000, 2001). The siRNAs are then incorporated into RISC, unwinding in the process (Rana, 2007). Synthetic siRNAs bypass this requirement for Dicer and enter the silencing pathway by directly incorporating into RISC following delivery into the cells (Rana, 2007) (Fig. 1-8).

The catalytic component of RISC, Argonaute-2 (Ago-2), cleaves the sense (passenger) strand of the siRNA, releasing it from the complex for subsequent degradation (Matranga *et al.*, 2005). The anti-sense (guide) strand of the siRNA guides the RISC complex to complementary mRNA sequences within the cell, and base-pairs with the target mRNA with perfect complementarity (Winter *et al.*, 2009). Argo-2 cleaves the corresponding target mRNA 10-11 nt upstream of the 5' end of the guide strand (Bernstein *et al.*, 2001; Hammond *et al.*, 2001). It is not clear how the activated RISC complex actually locates target mRNA. The cleaved mRNA is destroyed and cannot be used as a translation template, which leads to gene silencing (Rana, 2007). Expression of the gene of interest can be either almost completely or partially suppressed, depending on the amount of shRNA expressed and its potency (Bernstein *et al.*, 2001).

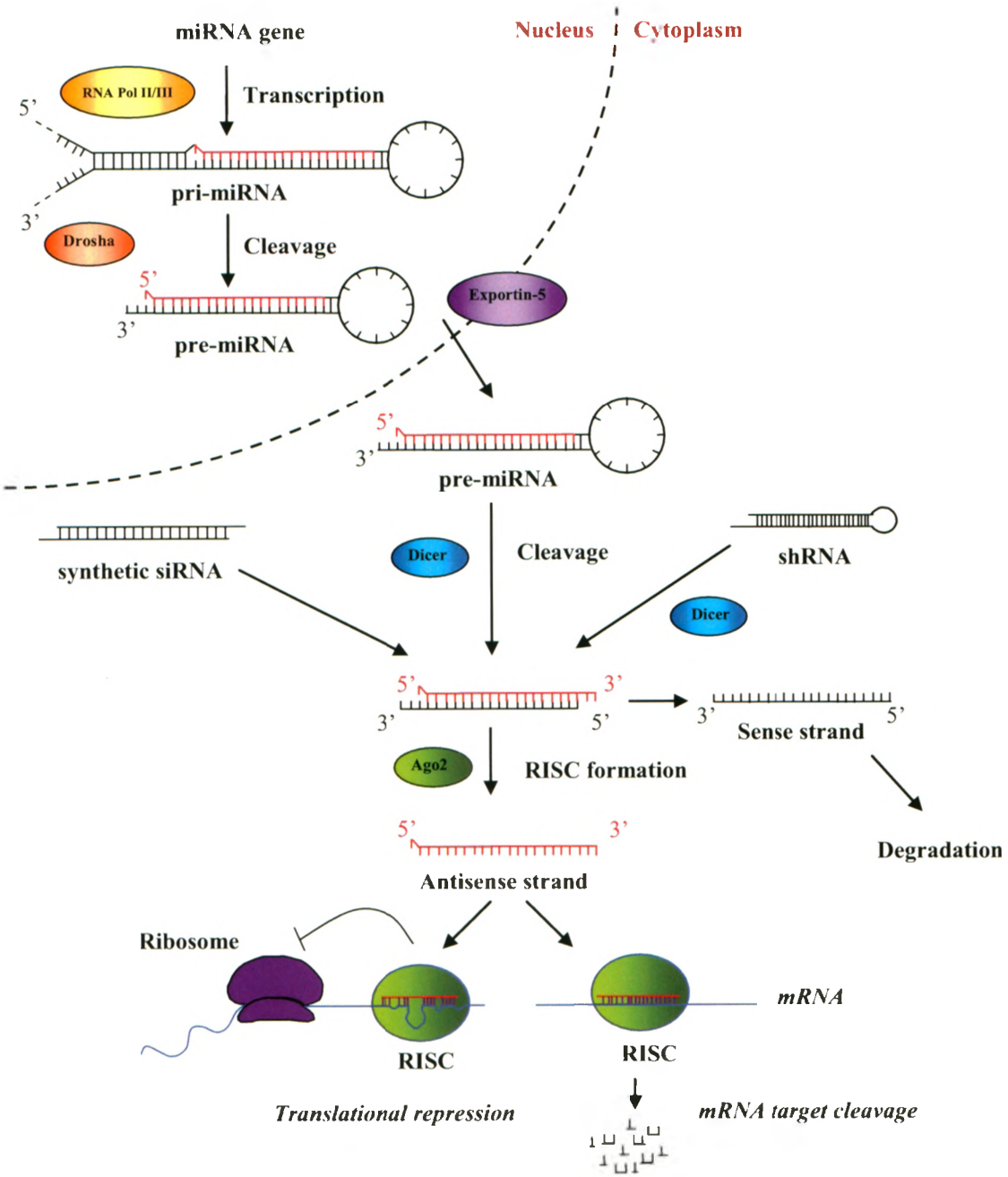

Fig. 1-8. RNAi-mediated silencing of gene expression. RNAi can be initiated in cells by the introduction of synthetic double stranded siRNA (left) or plasmid or viral vectors encoding shRNA (right) or by endogenously transcribed pri-miRNAs (middle). In the nucleus, Drosha processes the pri-miRNA into pre-miRNA which is exported into the cytoplasm by exportin-5. The common pathway for dsRNA, miRNA, and shRNA begins with Dicer cleavage of the intermediate transcripts to generate the 21-23 nt dsRNA. The duplexes unwind and the guide strand (red) is incorporated into RISC, where it guides RISC to silence target mRNA by either cleavage of the mRNA or translational repression depending on the degree of complementarity between the guide strand and the target mRNA. The passenger strand (black) is degraded. (*Adapted from Winter et al., 2009*)

1.7.4 Conventional versus miRNA-based shRNAs

The conventional stem-loop shRNAs described earlier are not processed by Drosha, but exported directly into the cytoplasm by exportin-5 and processed by Dicer into siRNA duplexes (Brummelkamp *et al.*, 2002a; Paddison *et al.*, 2002a). These are typically driven by RNA polymerase III promoters and contain stems of 19-29 nt with minimal flanking RNA sequence (Dickins *et al.,* 2005) (Fig. 1-9). Conventional shRNA vectors have been widely utilized by researchers due to their ease of construction (Manjunath *et al.*, 2009). However, over-expression of a U6-driven shRNA cassette was shown to interfere with natural cellular miRNA expression, which proved to be fatal for the mice used in the study (Grimm *et al.*, 2006). Additionally, siRNAs generated from these vectors have triphosphate at their 5′ ends and a variable number of overhangs at the 3′ end, making them sub-optimal substrates for exportin-5 and Dicer (Manjunath *et al.*, 2009).

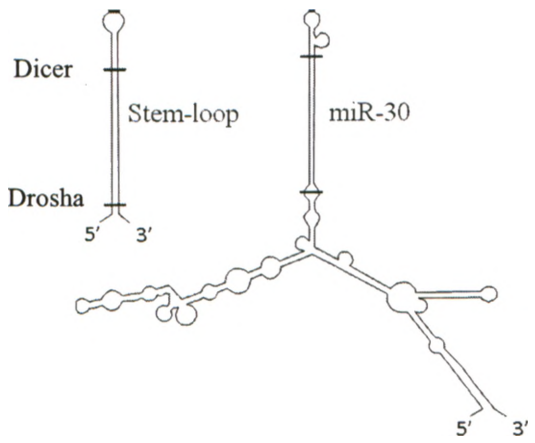

Fig. 1-9. Predicted RNA folding for conventional and miR-30-based shRNAs. Horizontal dashes indicate the approximate Drosha and Dicer cleavage sites. (*Adapted from Dickins et al., 2005)*

Investigators have recently turned to an alternative strategy, one based on the natural miRNA pathway (Fig. 1-10). In this pathway, Drosha cleaves the pri-miRNA to produce pre-miRNA with 2 nt overhangs at the 3' end (Manjunath *et al.*, 2009). Dicer recognizes this overhang and cuts the hairpin 22 nt away to produce the miRNA duplex which confers the silencing (Zhang *et al.*, 2002). In the natural miRNA pathway, therefore, processing by Drosha in the nucleus largely dictates the site of Dicer cleavage and which strand is incorporated into RISC (Matranga *et al.*, 2005). The idea behind the new strategy is to mimic the pri-miRNA processing to generate shRNAs. This was first attempted successfully by Zeng *et al.* (2002) who showed that replacement of the mature miR-30 stem region (~20 bp duplex) in the human miR-30 primary transcript with an artificial shRNA sequence led to specific and efficient gene silencing.

The addition of the miR-30 loop and 125 bases of the human miR-30 flanking sequence on either side of the hairpin has been shown to increase Drosha and Dicer processing of the expressed hairpins by 10-fold compared to the conventional designs, leading to higher siRNA production and a greater efficacy for expressed hairpins (Dickins *et al.*, 2005; Silva *et al.*, 2005). Artificial miRNA-based designs also offer more flexibility with respect to applications of gene silencing, being more amendable to RNA polymerase II transcription (allowing tissue-specific expression) and polycistronic strategies (permitting the delivery of multiple siRNA sequences) (Cai *et al.*, 2004; Chung *et al.*, 2006; Dickins *et al.*, 2005). Consequently, the use of artificial miRNAs as RNAi vectors has become an attractive alternative to traditional stem-loop shRNAs.

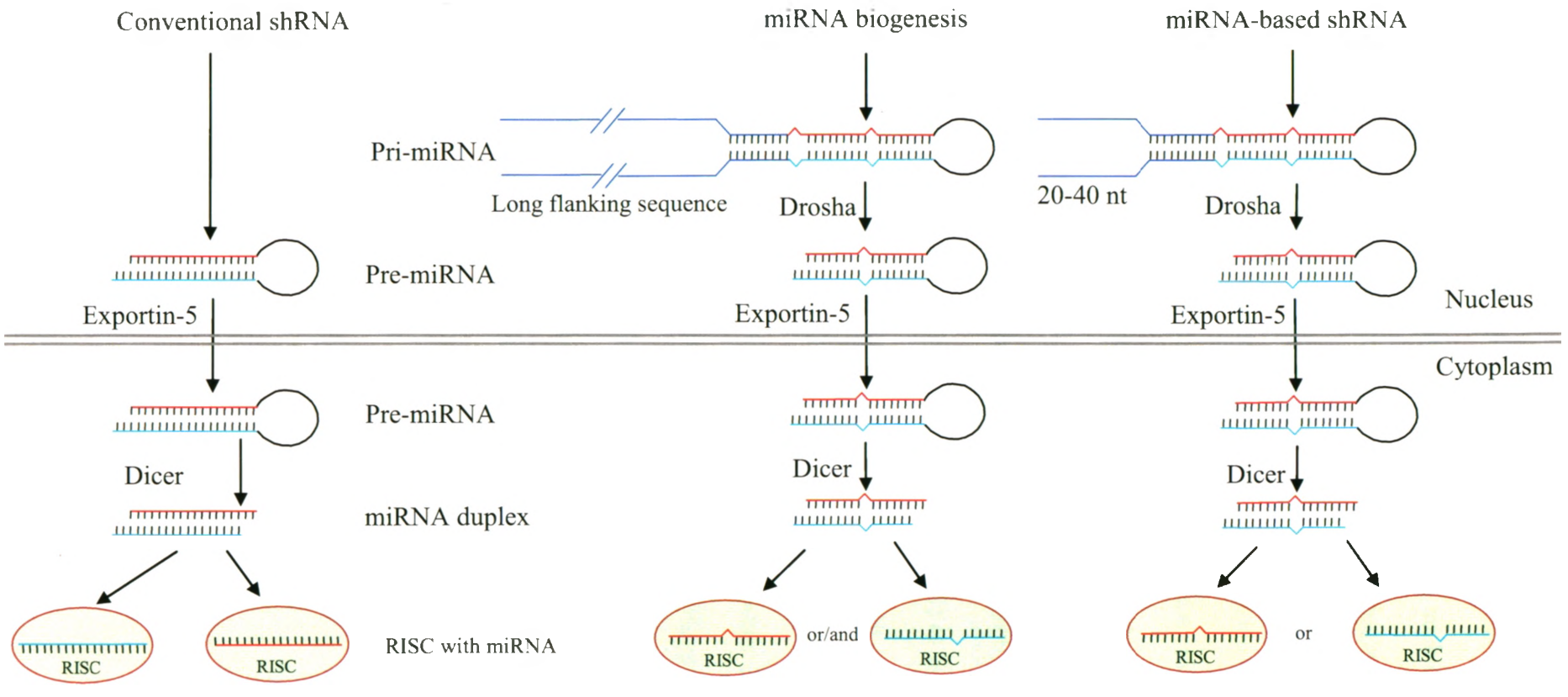

Fig. 1-10. Biogenesis of conventional and miRNA-based shRNAs. Conventional shRNAs (left) contain only the hairpin RNA sequence, which is directly exported to the cytoplasm. In the natural miRNA pathway (middle) the pri-miRNA transcript contains the hairpin in addition to a long flanking sequence, which is cleaved by Drosha to generate the pre-miRNA that is processed by Dicer into short RNA duplexes. In the miRNA-based shRNA strategy, the shRNA mimics the pri-miRNA by adding the miRNA flanking sequences to the shRNA stem, which allows it to be processed by the natural miRNA pathway. (*Adapted from Manjunath et al., 2009*)

1.7.5 Applications of RNAi for Silencing

Although genetically engineered animal models, such as knockout and transgenic mice, have many advantages, their development requires considerable time and resources. RNAi is changing the field of functional genomics by providing a more rapid and inexpensive means of suppressing target gene expression with high specificity (Rana, 2007). RNAi has been used to generate model systems, to identify novel molecular targets and to study gene function (Pushparaj *et al.*, 2008; Rao *et al.*, 2009).

RNAi-mediated gene silencing has been demonstrated successfully in mammalian cells (Brummerlkamp *et al.*, 2002a/b; Elbashir *et al.*, 2001a; Miyagishi *et al.*, 2002; Paddison *et al.*, 2002a/b; Sui *et al.*, 2002; Yu *et al.*, 2002) as well as in mice (Bitko *et al.*, 2005; Lewis *et al.*, 2008; Sioud and Sorensen, 2003; Song *et al.*, 2003; Sorensen *et al.*, 2003; Soutschek *et al.*, 2004; Zhang *et al.*, 1999) and non-human primates (Li *et al.*, 2005; Zimmermann *et al.*, 2006). Thus, RNAi technology holds a great potential in creating knockouts as well as selective gene silencing (Huppi *et al.*, 2005; Matzke and Birchler, 2005), and is emerging as a potential therapeutic strategy for various diseases in humans, including viral infections, cancer and degenerative diseases (Aigner, 2006; Bumcrot *et al.*, 2006; de Fougerolles, 2005; Kim *et al.*, 2007).

1.8 INTRODUCTION TO THESIS

1.8.1 Study Rationale

The results of our lab outlined in the previous sections can be summarized as follows: 1) S100A8 and S100A8 levels are coordinately elevated in CF mouse lungs; 2) up-regulation of S100A8 or S100A8/S100A9, but not S100A9 alone, correlates with the development and progression of a lung-specific inflammatory phenotype in B6-CF mice; 3) there is an inherent defect in the ability of CF lung epithelia and/or neutrophils and macrophages to down-regulate S100A8/S100A9 expression following tissue-infiltration at sites of inflammation; and 4) suppression of S100A8 expression *in vivo* may provide a potential therapeutic strategy for ameliorating the lung-specific disease in CF. These findings justify additional studies to determine the precise role of S100A8 in the severe inflammatory phenotype observed in CF lungs, particularly, the consequences of S100A8 inhibition. The aim of this study was to establish an *in vitro* model for shRNA-mediated suppression of S100A8 which can be exploited in future mouse studies.

1.8.2 Hypothesis and Objectives

It was ***hypothesized*** that murine S100A8 expression could be suppressed in mammalian cells using shRNA. The main ***objectives*** were to (i) establish a mammalian cell line expressing constant levels of murine S100A8; and (ii) demonstrate the knockdown of S100A8 expression *in vitro* using shRNA.

1.8.3 Prelude to Experiments

1.8.3.1 Assessing S100A8 Expression in P388D Cells

The literature was reviewed to identify a murine cell line expressing endogenous S100A8 for the *in vitro* knockdown experiments. Primary murine neutrophils would have been ideal candidates for this study; however, due to the transient life span of freshly isolated neutrophils, it would not be possible to maintain these cells in culture long enough to conduct the RNAi experiments. The macrophage-like mouse (tumor) cell line, P388D1, has been shown to express detectable levels of S100A8 using cytospin preparations stained by the immunoperoxidase method (Goebeler *et al.*, 1993). This cell line, isolated by Dawe and Potter (1957) from a methylcholanthrene-induced lymphoid neoplasm (P388) of a DBA/2 mouse, possesses the characteristics of normal macrophages, and has been used in research to study macrophage functions where pure populations are required (Koren *et al.*, 1975). This cell line was assessed for the expression of S100A8.

Treatment with pro-inflammatory mediators such as bacterial lipopolysaccharide (LPS) has been shown to up-regulate S100A8 expression in various murine macrophage cell lines (Hsu *et al.*, 2005; Hu *et al.*, 1996). For example, murine macrophage-like RAW 264.7 and WEHI 265 cells treated with an initial dose of LPS for 20 hrs and challenged with a second treatment of LPS for the same duration showed maximal induction of S100A8, while the untreated control cells expressed undetectable levels of S100A8 (Hu *et al.*, 1996). *In vivo*, S100A8 mRNA and protein levels are highly induced in mouse lungs in response to intravenous injections of LPS (Passey *et al.*, 1999a). The effect of LPS on S100A8 expression in P388D1 cells was also examined in this study.

1.8.3.2 Investigation of S100A8 Induction in 32D Cells

Since knockdown of protein levels is the relevant concern *in vivo*, identifying a cell system which would permit the investigation of S100A8 knockdown at the protein level was important. With the exception of neutrophils and trophoblasts *in vivo* (Passey *et al.*, 1999a), there were no other murine cells shown to constitutively express S100A8 protein. Stimulation seemed to be necessary to induce S100A8 protein production. This has been demonstrated in the murine myeloid 32D cell line by omitting interleukin-3 (IL-3) from and adding granulocyte colony stimulating factor (GCSF) to the growth medium (Nacken *et al.*, 2000). The 32D cell line represents a bone marrow line of mixed colonies, including erythroid cells, neutrophilic granulocytes, monocytes and macrophages. IL-3 supports the proliferation and promyeloic status of 32D cells, whereas the addition of GCSF (without IL-3) induces these cells to differentiate into the granulocytic lineage (Valtieri *et al.*, 1987), at which point the cells were shown to express both S100A8 and S100A9 (Nacken *et al.*, 2000). Therefore, the 32D cell line was used in this study to assess the possible induction of S100A8 expression.

1.8.3.3 Generating Stable S100A8 Cell Lines

Due to the unsuccessful attempts in finding a cell type expressing murine S100A8, it was proposed that stable cell lines expressing constant levels of S100A8 be generated. *In vitro* knockdown studies generally involve the suppression of constitutively-expressed genes to avoid erroneous results due to cell stress that may arise from the induction process or differences in the response of cells to stimulants. S100A8 expression has been shown to be up-regulated in response to cell stress (Endoh *et al.*, 2009; Grimbaldeston *et al.*, 2003). This is another reason why stable cell lines were

created. COS-7 cells, established in the 1960s from the kidney of an African green monkey (Jensen *et al.*, 1964), were readily available. Thus, this model system was chosen to test the knockdown efficacy of the shRNAs.

1.8.3.4 Generating Stable S100A8/S100A9 Cell Lines

Murine S100A8 and S100A9 have been shown to form non-covalent homodimers and/or heterodimer complexes (Nacken *et al.*, 2000; Raftery and Geczy, 1998). Moreover, exclusive expression of S100A8 or S100A9 has been documented in several studies in murine systems (Rahimi *et al.*, 2005; Xu *et al.*, 2001). To analyze the importance of dimerization for the stability of the two S100 proteins *in vitro*, COS-7 cells that were initially stably transfected with murine S100A8 were re-transfected (stably) with a plasmid encoding the murine S100A9 gene.

1.8.3.5 Knockdown of S100A8 with shRNA

This study exploits RNAi technology to suppress the expression of S100A8 in a COS-7 cell line stable for S100A8 expression. The project was undertaken to establish a working *in vitro* model for S100A8 down-regulation by miR-30-based shRNAs in mammalian cells and to illustrate the potential of this knockdown system for studying S100A8 gene function *in vivo*; in particular, the consequences of S100A8 suppression on the inflammatory lung phenotype in CF mouse models.

2. MATERIALS & METHODS

2.1 GENERAL CELL CULTURE

2.1.1 Cells and Culture Media

Cells were grown in the appropriate culture medium (as described below) and maintained at 37°C in 5% CO_2. The murine myelomonocytic cell line, P388D1, was obtained from the American Tissue Culture Collection (ATCC) (Cat. No. CCL-46). P388D1 cells were cultured in DMEM (ATCC, Cat. No. 30-2002) with 4 mM L-glutamine, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, and supplemented with 10% horse serum (ATCC, Cat. No. 30-2040). The murine bone marrow 32D cell line was obtained from ATCC (Cat. No. CRL-11346) and cultured in RPMI-1640 medium (ATCC, Cat. No. 30-2001) with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, and supplemented with 1X antibiotic/antimycotic solution [10 000 I.U. Penicillin (per ml), 10 000 μg/ml Streptomycin, 25 μg/ml Amphotericin B, and 8.5 g/L NaCl] (Wisent, Cat. No. 450-115-EL), 10% heat-inactivated fetal bovine serum (FBS) (Wisent, Cat. No. 080150), and 10% mouse Interleukin-3 (IL-3) (Sigma, Cat. No. I-4144) (this cell line is IL-3 dependent). FBS was heat-inactivated by heating to 56°C for 30 min to inactivate complement. The COS-7 (African green monkey kidney) fibroblast-like cell line was kindly provided by Dr. Joe Mymryk (London Regional Cancer Program, London, Ontario, Canada). Cells were cultured in DMEM (Wisent, Cat. No. 319-020-CL) with 4.5 g/L L-glutamine, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and supplemented with 1 mM sodium pyruvate, 1X antibiotic/antimycotic solution (Wisent, Cat. No. 450-115-EL), and 10% FBS (Wisent, Cat. No. 080150).

2.1.2 Sub-culturing Cells

Adherent COS-7 cells grown to confluence were rinsed twice in Dulbecco's phosphate-buffered saline (DPBS) (Wisent, Cat. No. 311-425-CL), and detached by incubating with an appropriate amount of trypsin-ethylene-diamine-tetraacetate (EDTA) solution (0.05% trypsin and 0.02% EDTA w/v in DPBS) (Wisent, Cat. No. 325-043-EL) for 4-5 min at 37°C. Complete culture medium (with serum) was added to arrest further tryptic digestion, and cells were centrifuged and seeded into 10 cm Petrie dishes for maintenance, or onto wells of six-well plates for experimentation. Suspension cell lines, P388D1 and 32D, were cultured in 25 cm^2 flasks in the appropriate growth media. For the 32D cell line, cell scrapers were used to detach adherent cells in this mixed cell population. Cells were sub-cultured every 2-3 days.

2.1.3 Cell Cryopreservation and Revival

For storage, all cell lines were suspended in 5% dimethylsulphoxide (DMSO) (Sigma, Cat. No. D2650) in the appropriate culture medium. Aliquots of 1 ml were dispensed into 2 ml sterile Cryo tubes which were kept at -80°C overnight and then transferred to -150°C for long-term storage. To thaw out cells, tubes containing frozen cells were taken from -150°C and rapidly (<2 min) thawed at 37°C, and transferred into Falcon tubes containing 10 ml of the appropriate culture medium warmed to 37°C and centrifuged at 125 x g for 5 min at room temperature. Cells were re-suspended in fresh culture medium containing 20% serum (to help cell recovery) and seeded into the appropriate vessel. After 4-6 hrs, the culture medium was replaced with fresh growth medium containing 10% serum.

2.2 GENERATING STABLE CELL LINES

2.2.1 Transformations and Plasmid Purification

All DNA vectors used in this study (pDream2.1/S100A8, pcDNA3.1/S100A9, and MSCV-LTRmiR30-PIG/shRNA vectors) were transformed into DH5α competent cells. Briefly, 1.5 µl of plasmid DNA was mixed with 50 µl of DH5α cells (Invitrogen, Cat. No. 18258-012) in a pre-chilled 1.7 ml Eppendorf tube, and incubated on ice for 30 min. The cells were subjected to heat-shock at 42°C for 45 sec and returned to ice for an additional 5 min. SOC medium (450 µl) was added to the tube followed by incubation at 37°C with shaking (225 rpm) for 1 hr. The cell suspension was then diluted to different concentrations (1:10, 1:50, 1:100, 1:250, 1:500, and 1:1000) and 100 µl of cells were spread onto pre-warmed LB plates with 100 µg/ml ampicillin and incubated at 37°C for 16 hrs. Then, 150 ml LB+ampicillin (100 µg/ml) liquid media was inoculated with individual bacterial colonies and incubated at 37°C with shaking (250 rpm) for 16 hrs. Plasmid DNA was purified using a QIAGEN Plasmid Maxi Prep Kit (Cat. No. 12162), according to the manufacturer's protocol. DNA was re-dissolved in TE buffer, pH 8.0.

2.2.2 Determination of Plasmid Yield

To determine the yield, plasmid concentration was determined by both UV spectrophotometry (Fisher Scientific, Beckman Model No. DU-530) at 260 nm as well as quantitative and qualitative analysis on an ethidium bromide-stained agarose gel. A_{260} readings between 0.1 and 1.0 were considered reliable. For restriction enzyme digestion, 1 µl of the appropriate enzyme was mixed into 1 µg of plasmid DNA, 2 µl of appropriate 10X reaction buffer, and 16 µl of H_2O at the enzyme's optimal temperature and duration, and run on a 1% agarose gel to confirm the purity of the isolated plasmid.

2.2.3 Transfecting COS-7 Cells with Murine S100A8

COS-7 cells were seeded into six-well plates 24 hrs before transfection, giving 90-95% confluence on the day of transfection. Cells were transfected with the pDream2.1 expression vector (GenScript, Cat. No. SD0221) (Fig. 2-1) containing the murine S100A8 gene (GenBank Accession No. NM_013650) using Lipofectamine™ 2000 (Invitrogen, Cat. No. 11668-019) according to the manufacturer's recommendations. The S100A8 sequence cloned into pDream2.1 (7156 bp) is 270 bp in length, and represents the coding region of the mouse gene. Cloning and sequence verification were carried out by GenScript, and confirmed again by our lab. Stable clones were obtained after six weeks of growth with 600 μg/ml geneticin-G418 (Wisent, Cat. No. 450-130-QL). Reverse-transcriptase PCR (RT-PCR) (Section 2.5) and Western blotting (Section 2.7) were used to detect S100A8 mRNA and protein levels, respectively.

2.2.4 Re-transfecting Stable COS-7 Cells with Murine S100A9

To generate stable cell lines expressing both S100A8 and S100A9, pcDNA3.1/Hygro(+) expression vector (Sigma, Cat. No. V870-20) (Fig. 2-2) containing the murine S100A9 gene (GenBank Accession No. NM_009114) was introduced into the stable S100A8 COS-7 cells by transfection with Lipofectamine™ 2000 (Invitrogen, Cat. No. 11668-019). The S100A9 sequence cloned into pcDNA3.1/Hygro(+) (5597 bp) is 342 bp, and represents the coding region of the mouse gene. Cloning and sequence verification were carried out by GenScript. Both transient and stable transfection experiments were performed. Stable cell lines were obtained after six weeks of selection with 100 μg/ml hygromycin (Wisent, Cat. No. 450-140-XL). Once independent colonies became visible, they were sub-cultured separately until ready to assay.

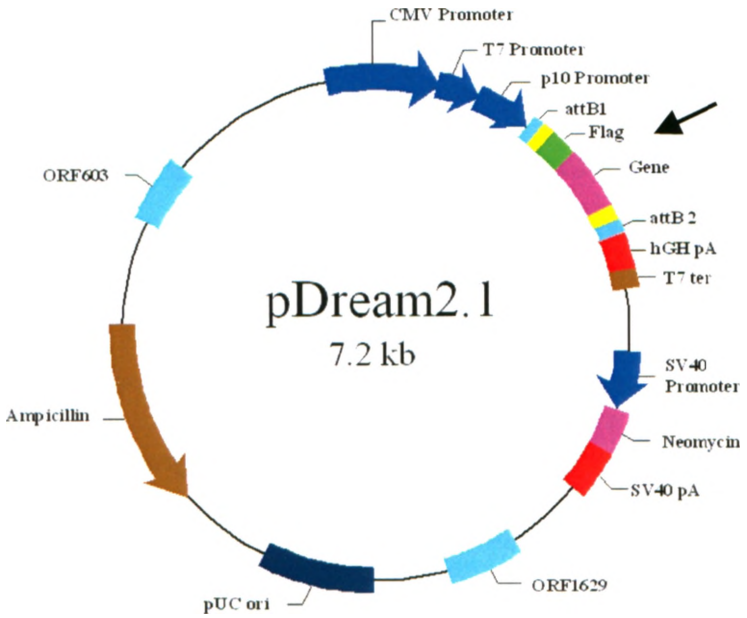

Fig. 2-1. Vector map of pDream2.1 expression vector (7156 bp). Arrow indicates the cloning region of murine S100A8. *(Adapted from GenScript)*

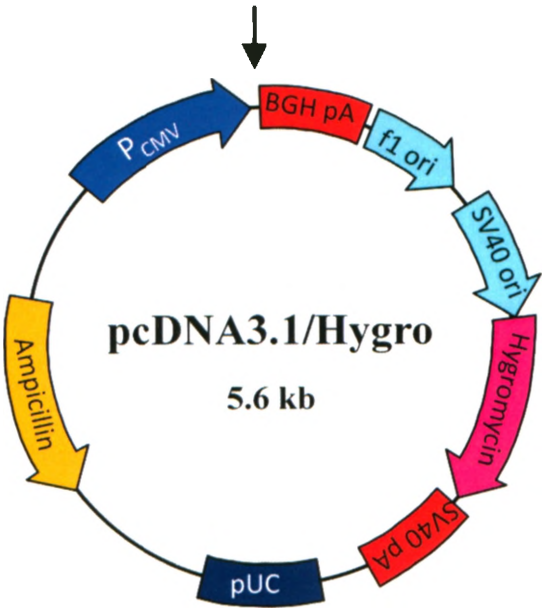

Fig. 2-2. Vector map of pcDNA3.1/Hygro(+) expression vector (5597 bp). Arrow indicates the cloning region of murine S100A9. (*Adapted from Sigma-Aldrich)*

2.3 CELL TREATMENTS

2.3.1 Treating P388D1 Cells with LPS

LPS from *Escherichia coli*, 055:B5, was obtained from Sigma (Cat. No. L-6529). P388D1 cells were initially treated with one dose of LPS at 100, 500, or 1000 ng/ml in 10 ml of growth medium. Cells were incubated for 20 hrs with LPS and collected thereafter for RT-PCR analysis. In the second experiment, cells were first primed with an initial dose of LPS (10 ng/ml) for 20 hrs. The medium was then changed with fresh growth medium and the cells challenged with a second dose of LPS (100, 500, or 1000 ng/ml) for an additional 20 hrs. Cells were harvested by centrifugation at 1000 x g for 5 min. RNA collection and RT-PCR was performed as described in Section 2.5.

2.3.2 Treating 32D Cells with GCSF

Mouse granulocyte colony-stimulating factor (GCSF) was obtained from Sigma (Cat. No. G8160) as a powder and reconstituted with sterile (0.2 μm-filtered) water to a stock concentration of 100 μg/ml. According to the study by Nacken *et al.* (2000), differentiation of 32D cells was induced by omitting IL-3 and the addition of 60 pg/ml of GCSF to the culture medium. Expression of S100A8 and S100A9 was monitored by Western blotting of cell lysates. To analyze the possible secretion of S100A8 into the medium, the supernatant of GCSF-treated 32D cells was collected and proteins precipitated with ethanol. Briefly, 1 volume of protein solution was added to 9 volumes of cold 100% ethanol, mixed and kept at -20°C for 1 hr. The supernatant was subjected to centrifugation for 15 min at 13000 x g, and the pellet was air-dried, washed with 90% cold ethanol, centrifuged again for 5 min, and re-suspended in a minimal volume of SDS-PAGE loading buffer. Western blotting was then performed on the precipitate.

2.3.3 Treatment of Stable COS-7 Cells with MG-132

To examine the possible degradation of the S100A8 protein, COS-7 cells stable for S100A8 or S100A8/S100A9 expression were treated with MG-132 (Calbiochem, Cat. No. 474791) at 10 μM for 4 hrs or 50 μM for 2 hrs prior to collection for protein analysis.

2.4 KNOCKDOWN OF S100A8 WITH SHORT-HAIRPIN RNA

2.4.1 Design Criteria for shRNAs

There are several parameters which must be considered for the efficient design of shRNA knockdown systems. The selection of target sequences for knockdown studies represents the first critical step that governs the success of the experiment (Elbashir *et al.*, 2001a). Several algorithms and criteria have been published in this regard (Amarzguioui and Prydz, 2004; Elbashir *et al.*, 2001a/b; Hsieh *et al.*, 2004; Reynolds *et al.*, 2004; Saetrom *et al.*, 2004; Takasaki *et al.*, 2004; Ui-Tei *et al.*, 2004, 2007). Generally, several different shRNA molecules must be designed and tested to identify effective silencers. There are a number of web-based programs that assist in selecting siRNA/shRNA sequences on the basis of the highest predicted knockdown efficiency, including "The siRNA Target Finder and Design Tool" by Ambion, (www.ambion.com/techlib/misc/siRNA_finder.html), "*siDESIGN* Center" by Dharmacon (www.dharmacon.com/DesignCenter/DesignCenterPage.aspx), and "BLOCK-iT™ RNAi Designer" by Invitrogen (https://rnaidesigner.invitrogen.com/rnaiexpress/). Introns and 5' and 3' untranslated regions (UTRs) are usually avoided, as well as repeats of 4 or 5 A's or T's for siRNA sequences to be expressed from an RNA polymerase III promoter (repeats can act as termination sites), with the target being 50 to 100 nt downstream of the start codon (Elbashir *et al.*, 2001b; Reynolds *et al.*, 2004; Ui-Tei *et al.*, 2004, 2007).

The GC content of the molecule is also critical in determining its efficacy (Reynolds *et al.*, 2004). Once the candidate sequences are chosen, a search of the genome database BLAST (www.ncbi.nlm.nih.gov/BLAST) is performed to make sure that the shRNA targets a single gene.

General guidelines for siRNA design are provided below (obtained from www.protocol-online.org/prot/Detailed/3210.html):

1 - Targeted sequence is usually 21 nt in length.

2 - Avoid regions within 50-100 bp of the start codon and the termination codon

3 - Avoid intron regions

4 - Avoid stretches of 4 or more bases such as AAAA, CCCC

5 - Avoid regions with GC content <30% or > 60%.

6 - Avoid repeats and low complex sequence

7 - Avoid single nucleotide polymorphism (SNP) sites

Reynolds *et al.* (2004) published eight criteria for effective siRNA design, including a number of position-specific nucleotide preferences and other siRNA features, as follows:

1 - Moderate to low (30%-52%) GC content

2 - At least 3 'A/U' bases at positions 15-19 (sense strand)

3 - Absence of internal repeats (temperature of potential internal hairpin < 20°C)

4 - 'A' at position 19 (sense strand)

5 - 'A' at position 3 (sense strand)

6 - 'U' at position 10 (sense strand)

7 - No 'G' or 'C' at position 19 (sense strand)

8 - No 'G' at position 13 (sense strand)

The optimal length of the shRNA stem and loop varies among researchers. Stem lengths of 19-29 bp and loop sizes ranging between 3-23 bp have been reported to function well in gene silencing studies (Brummerlkamp *et al.*, 2002; Lee *et al.*, 2002; Miyagishi *et al.*, 2002; Sui *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Yu *et al.*, 2002). Furthermore, the thermodynamic stability at the 5' end of the antisense strand seems to play a vital role in the silencing efficacy of the molecule. To ensure that the antisense (guide) strand is preferentially used by RISC, shRNAs are designed to have lower thermodynamic stability at the 5' terminus of the antisense strand by introducing mismatches of nucleotides (asymmetry) at this end of the molecule (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003). This allows for the proper directional loading of siRNA to ensure stable incorporation of the antisense instead of the sense strand into RISC and reduces the likelihood of off-targeting by the sense strand (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003).

2.4.2 shRNA Constructs

Three different shRNA molecules with the appropriate nucleotide modifications targeted to murine S100A8 as well as a non-relevant shRNA control (targeting the murine CLCA3 gene) were generated in consultation with scientists at, and synthesized by, Sigma-Genosys, according to established protocols (Dickins *et al.*, 2005; Silva *et al.*, 2005). These were named shRNA_mA8_65, shRNA_mA8_250, shRNA_mA8_340 and shRNA_mCLCA3_665 based on their starting nucleotide positions within the coding region of the target mRNA, corresponding to the 3' shRNA antisense end (Table 2-1). The shRNA sequences were cloned into the MSCV-LTRmiR30-PIG expression cassette, driven by the murine stem cell virus (MSCV) 5' long terminal repeat (LTR), a strong

RNA polymerase II promoter (Dickins *et al.*, 2005) (Fig. 2-3). The shRNA cassette also expresses a green fluorescent protein (GFP) gene driven predominantly by the 5'LTR promoter (and to a smaller degree by a PGK promoter driving the expression of the puromycin resistance gene).

Table 2-1. Short-hairpin RNA target sequences

Accession	Gene	shRNA	Target Sequence
[GenBank: NM_013650]	Murine S100A8	shRNA_mA8_65	CCTTGAGCAACCTCATTGA
	Murine S100A8	shRNA_mA8_250	GAGTTCCTTGCGATGGTGA
	Murine S100A8	shRNA_mA8_340	CTGGATATGTCTACAGAAT
[GenBank: NM_017474]	Murine CLCA3	shRNA_mCLCA3_665	GGGACTGTATAAAGACAAT

The design of the hairpins was based on the human miR-30 microRNA. In miRNA-based shRNA expression vectors, the shRNA construct is designed to mimic a natural pri-miRNA by including the miRNA flanking sequence (125 bp) into the shRNA stem on either side of the hairpin. The resulting transcript is processed like miRNA by Drosha, followed by Dicer into siRNA (Manjunath *et al.*, 2009). The hairpin sequence is 97 bp in length, which, after processing by Dicer, generates a mature 19-mer product. Figure 2-4 shows the target region of each shRNA in murine S100A8 cDNA. These shRNAs were pre-designed but their suppressive activity was not validated.

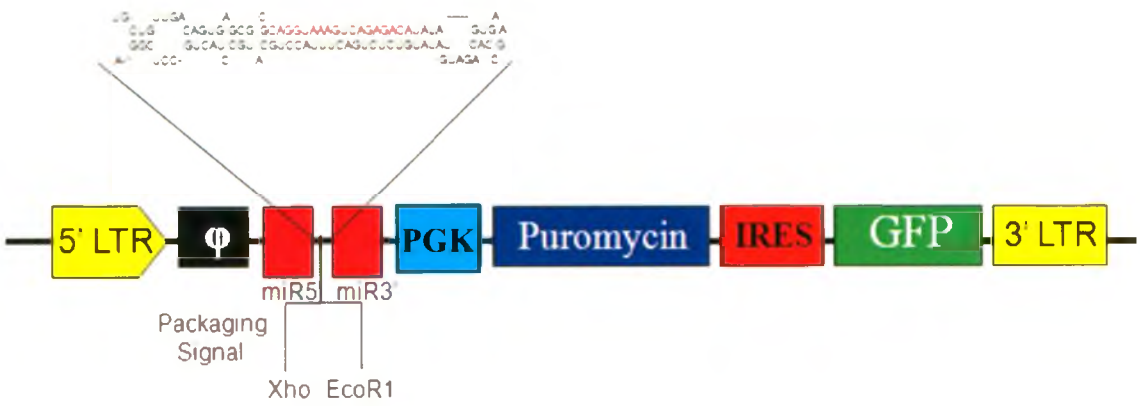

Fig. 2-3. MSCV-LTRmiR30-PIG shRNA vector construct (7894 bp). The 5'LTR promoter drives the expression of the shRNA and GFP. The PGK promoter drives the expression of the puromycin resistance gene. *(Adapted from Dr. Michael Golding)*

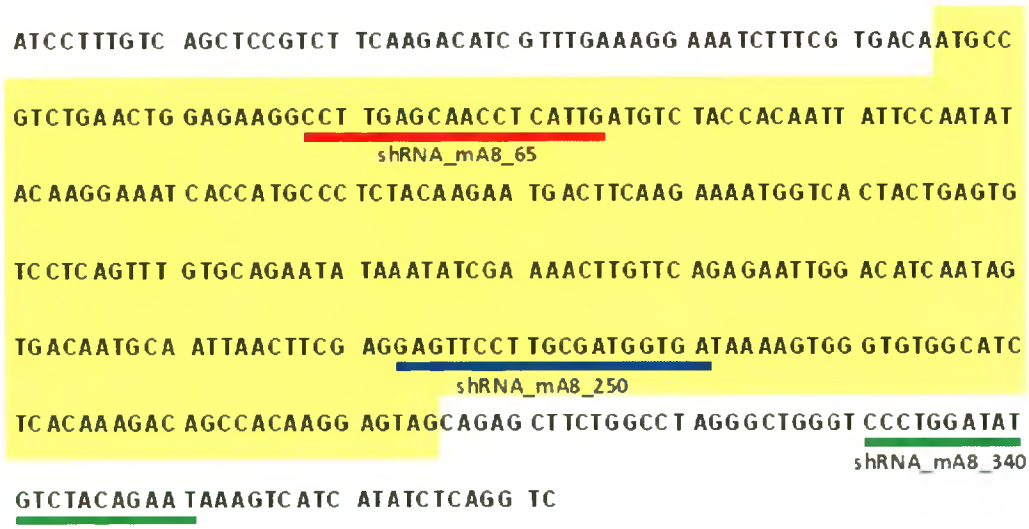

Fig. 2-4. Target regions of shRNAs in murine S100A8 cDNA. shRNA_mA8_65 (red underline); shRNA_mA8_250 (blue underline); shRNA_mA8_340 (green underline). The 270 bp coding region of murine S100A8 (bases 56 to 325) is highlighted in yellow. Note that shRNA_mA8_340 target sequence lies in the 3' UTR of the S100A8 gene.

2.4.3 Sequence Alignment of Mouse S100 genes

To confirm that the shRNAs used in this study are specific for murine S100A8 and would not target other members of the S100 gene family, a sequence alignment analysis of all known mouse S100 genes was performed using the ClustalW2 (EMBL-EBI) web-based alignment program (www.ebi.ac.uk/Tools/clustalw2/index.html).

2.4.4 Generating Stable shRNA Cell Lines

The MSCV-LTRmiR30-PIG shRNA vectors designed against murine S100A8 and the non-specific (control) shRNA were used to measure and compare the level of S100A8 knockdown. All four shRNAs were separately transfected into the stable S100A8-expressing COS-7 cell line using Lipofectamine™ 2000 (Invitrogen, Cat. No. 11668-027) according to the manufacturer's instructions. Briefly, one day before transfection, cells were plated with 2 ml culture medium into six-well plates, such that they were 90-95% confluent at the time of transfection. For each well, 2 μg of plasmid DNA and 4 μl of Lipofectamine™ 2000 were diluted separately in 100 μl serum-free DMEM, and incubated for 10 min at room temperature. The two solutions were combined and incubated for an additional 20 min to allow the lipid-DNA complexes to form. The lipid-DNA mixture (200 μl) was added to each experimental well (containing 600 μl of serum- and antibiotics-free growth media), and plates incubated for 24 hrs at 37°C. Control wells received Lipofectamine™ 2000 reagent only. Transient transfections had very poor efficiency; therefore, it was necessary to create stable shRNA cell lines. Stable clones were obtained seven weeks post-transfection with puromycin (Wisent, Cat. No. 400-160-EM) at 10 μg/ml. G418 selection was continued to maintain S100A8 expression in these cells. When independent colonies became visible, they were cultured

separately until ready to assay for gene knockdown. Six different stable clones for each shRNA were analyzed for S100A8 mRNA levels by real-time RT-PCR (Section 2.6), and four of these clones (per shRNA treatment) were also evaluated for GFP expression.

2.4.5 Fluorescence Microscopy

Bright field and fluorescence images of stable shRNA cells were taken with the Olympus IX-70 inverted fluorescent microscope (Magnification: x100), captured with an Olympus DP71 digital camera using Image-Pro Plus 6.2 software (Media Cybernetics).

2.5 RNA PURIFICATION

2.5.1 RNA Extraction

Total RNA was isolated using TRI-Reagent (Sigma, Cat. No. T9424) according to the manufacturer's instructions. In brief, adherent cells grown as monolayers were lysed directly in six-well plates with 1 ml TRI-Reagent and homogenized samples were incubated for 5 min at room temperature. Suspension cells were pelleted by centrifugation and lysed in 1 ml TRI-Reagent. Cell lysates were transferred to Eppendorf tubes and 0.2 ml of chloroform (EMD, Cat. No. CX1054-6) was added to each sample and tubes were vigorously shaken by hand for 15 sec and incubated at room temperature for 2-3 min. Samples were then centrifuged at 12000 x g for 16 min at 4°C. The colorless upper aqueous layer containing RNA was transferred to a fresh tube. Total RNA was precipitated by mixing with 0.5 ml isopropyl alcohol, and samples were incubated at room temperature for 10 min, and then centrifuged at 12000 x g for 10 min at 4°C. RNA pellets were washed once with 1 ml 75% ethanol and centrifuged at 7500 x g for 5 min at 4°C. RNA pellets were air-dried for 10 min, and re-dissolved in RNase-free water at

50°C for 10 min. Quantification was determined by spectrophotometry (Fisher Scientific, Beckman Model No. DU-530) at A_{260}/A_{280}. A ratio of 1.6 to 1.9 was considered reliable.

2.5.2 Reverse-Transcription (RT) Reaction

RNA samples were treated with amplification grade DNase I (GenScript, Cat. No. S2222) according to the manufacturer's protocol. DNAse I-treated total RNA (1 μg) was reverse-transcribed using the SuperScript™ II RNase H-Reverse Transcriptase kit (Invitrogen, Cat. No. 18064-022). Total RNA, 10 mM dNTP mix, and random primers (250 μg/ml) were heated at 65°C for 5 min, and then chilled on ice. This was followed by the addition of 5X First-Strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$), 0.1 M DTT, and 40 units of RNaseOUT (Invitrogen, Cat. No. 10777-019) and incubation at 25°C for 2 min. Then, 1 μl (200 units) of SuperScript™ II RT was added and samples were incubated for an additional 10 min at 25°C, followed by 50 min at 42°C. Negative controls (no first strand synthesis) were prepared by performing reverse transcription reactions in the absence of the reverse transcriptase enzyme. Reactions were heat-inactivated at 70°C for 15 min. To remove RNA templates, 1 μl (2 units) RNase H (Invitrogen, Cat. No. 18021-071) was added to each tube and incubated at 37°C for 20 min. The cDNA was stored at -20°C.

2.5.3 Polymerase Chain Reaction (PCR)

PCR was performed for 30 cycles (30 sec at 94°C, 30 sec at 59°C, and 30 sec at 72°C, followed by a 10 min elongation period at 72°C) using the Gene Amp PCR System 9700 (Perkin Elmer Applied Biosystems, Model No. N8050200), and standard reaction conditions: 2 μl cDNA, 2 mM dNTPs, 10X reaction buffer (pH 9.0) (GenScript, Cat. No.

B0005), 2 units of Taq DNA polymerase (GenScript, Cat. No. E00007), and 50 ng/μl of each S100A8 primer (Table 2-2). PCR products were analyzed on an ethidium bromide-stained 1.5% agarose gel and visualized by Fugifilm Thermal Imaging System FTI-500 (Pharmacia Biotech, Model No. 80-6246-82). The expected S100A8 amplification product was 264 bp. PCR for β-actin verified the loading of equal template amounts (primer sequences in Table 2-2). cDNA from mouse bone marrow (BM) and mock RT reactions served as positive and negative controls, respectively. A 1 Kb Plus DNA Ladder™ (Invitrogen, Cat. No. 10787-018) was used for sizing the DNA products.

Table 2-2. List of gene-specific primers*.

Gene	Forward Primer	Reverse Primer
Mouse S100A8	5'-GCCGTCTGAACTGGAGAA -3'	5'-TCCTTGTGGCTGTCTTTG-3'
GFP	5'-GTGACCACCCTGACCTACGG-3'	5'-GATGTTGTGGCGGATCTTGA-3'
Mouse GAPDH	5'-AACGACCCCTTCATTGAC-3'	5'-TCCACGACATACTCAGCA-3'
Mouse β-Actin	5'-TCGTGGGCCGCTCTAGGCACCA-3'	5'-GTTGGCCTTAGGGTTCAGGGGGG-3'

**All primer sets were supplied by Sigma-Aldrich*

2.6 REAL-TIME RT-PCR

Real-time quantitative RT-PCR (qRT-PCR) analysis was used to compare S100A8 and GFP mRNA expression levels between the stable shRNA cell lines. S100A8 forward and reverse primers were used at 100 ng/μl and 200 ng/μl, respectively (Table 2-2). Thermal cycling was carried out for 35 cycles (94°C for 30 sec, 60°C for 60 sec and 77°C for 30 sec) using the MX300P QPCR System (Stratagene, Cat. No. 401400). GFP expression was analyzed using forward and reverse primers at 100 ng/μl and 300 ng/μl, respectively (Table 2-2). Thermal cycling for GFP analysis was carried out for 35 cycles

(94°C for 30 sec, 67°C for 60 sec, and 72°C for 30 sec). Standard curves for each sample were determined using serially diluted cDNA (1:5, 1:25, 1:125) to ensure primers and conditions are optimal. The melting temperature profile of the reaction products from the standard curve was determined to ensure the desired product is being measured and that non-specific products were absent. All PCR reactions were performed in duplicate, using Brilliant SYBR Green Reagent (Stratagene, Cat. No. 600548-51) and analyzed in 96-well optical reaction plates. Comparative quantitation was performed on cDNA from wild-type COS-7 cells, S100A8-transfected (stable) cells, and S100A8+shRNA-transfected (stable) cells. A 1:25 dilution of cDNA was used for each sample. Relative quantities of mRNA expression levels were computed using the comparative Ct (threshold cycle) method and normalized against β-actin. Negative control samples (no first strand synthesis) were prepared by performing reactions without reverse transcriptase. Values are represented as means ± SEM (N=6 per group for S100A8; N=4 per group for GFP).

2.7 WESTERN BLOT ANALYSIS

Cell lysates were analyzed for the presence of the two S100 proteins by Western blotting. Cells were lysed with a standard lysis buffer (containing 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1% TritonX-100, and 1% NP-40 dissolved in water) with Complete Protease Inhibitor Cocktail (Sigma, Cat. No. P8340). Cell lysates were centrifuged at 13,000 rpm for 10 min at 4°C. Protein concentrations were measured comparatively with BSA standards. BSA standards were determined using the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Cat. No. 500-0112) according to the manufacturer's instructions. Plates were read at 655 nm using Model 680 Microplate Reader and the Microplate Manager 5.2.1 Software (Bio-Rad Laboratories, Hercules,

CA). Total protein samples (50-100 μg) were mixed with SDS loading dye, heated to 95°C for 5 min, and subjected to 15% SDS-PAGE for 2 hrs at 100 V according to standard protocols. Blots were then transferred onto polyvinylidene difuoride (PVDF) membranes (GE Healthcare, Cat. No. RPN303F) for 1 hr at 90 V. Membranes were blocked overnight with 5% (w/v) non-fat skim milk in phosphate-buffered saline containing 0.05% (v/v) Tween-20 (PBST) at 4°C. Membranes were incubated with a 1:1000 (v/v) dilution of anti-mouse S100A8 (Santa Cruz Biotechnology, Cat. No. sc-8113) or anti-mouse S100A9 (Santa Cruz Biotechnology, Cat. No. sc-8115) 1° antibody in 5% (w/v) skim milk in PBST for 2 hrs at room temperature. Blots were washed with PBST and incubated with a 1:400,000 (v/v) dilution of horseradish peroxidase-conjugated 2° rabbit anti-goat antibody (Pierce, Cat. No. 31402) in 5% (w/v) skim milk in PBST at room temperature for 1 hr with gentle agitation. Primary anti-rabbit β-actin (Santa Cruz, Cat. No. sc-1616-R) and 2° goat anti-rabbit (Pierce, Cat. No. 1858415) antibodies were used at dilutions of 1:2000 and 1:100,000, respectively, to detect β-actin to confirm equal loading of protein. Target proteins were visualized with the Pierce SuperSignal System (Thermo Scientific, Cat. No. 34095). Western blots were developed using the Kodak M35A X-OMAT Processor (Kodak Diagnostic Imaging).

2.8 STATISTICAL ANALYSIS

Values in figures are expressed as means ± SEM. Student's T-tests (two-tailed, unpaired) were performed to compare relative S100A8 expression levels between shRNA treated and untreated cells. A one-way analysis of variance (ANOVA) was performed to compare relative GFP expression levels between the different shRNA treatments. A value of $P<0.05$ was considered significant.

3. RESULTS

3.1 ANALYSING P388D CELLS FOR S100A8 EXPRESSION

P388D1 cells were previously reported to express S100A8 (Goebeler *et al.*, 1993). However, RT-PCR analysis did not reveal S100A8 expression in unstimulated P388D1 cells (Fig. 3-1). Cells were then challenged with 100, 500, or 1000 ng/ml of LPS for 20 hrs, following a primary exposure to 10 ng/ml of LPS. Again, no S100A8 mRNA transcripts were detected with RT-PCR (Fig. 3-1). Because the degree of confluency may affect induction of S100A8 by LPS, S100A8 expression was analyzed in cells grown at low (40-50%) and high (95-100%) densities and stimulated with priming (10 ng/ml LPS for 20 hrs) and challenge (500 ng/ml LPS for 20 hrs) doses of LPS. However, both treatment regimes did not induce S100A8 expression (Fig. 3-2).

This cell line was also tested to see if priming alone (at 5 or 10 ng/ml LPS) or challenge alone (at 100, 500, or 1000 ng/ml LPS) could induce expression of S100A8. However, the expected 264 bp S100A8 product was not observed in any treatment (Fig. 3-3). The positive (mouse bone marrow) control confirmed the competence of the S100A8 primers used in the RT-PCR reactions. Thus, LPS-stimulation failed to induce the expression of S100A8 in the P388D1 cell line.

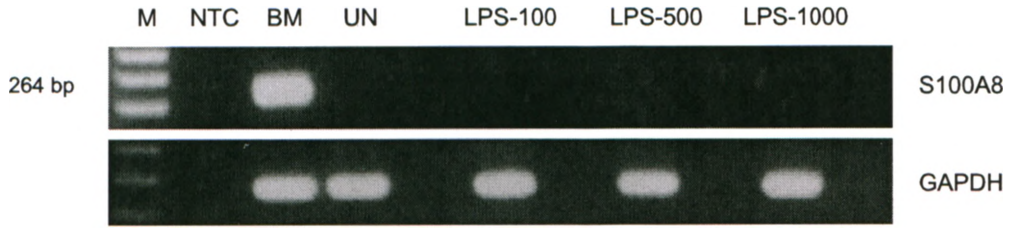

Fig. 3-1. RT-PCR analysis of S100A8 expression in untreated and LPS-stimulated P388D1 cells. *Upper panel*: P388D cells showed no expression of S100A8 mRNA without stimulation (UN), or when primed (10 ng/ml for 20 hrs) and challenged for an additional 20 hrs with 100 ng/ml (LPS-100), 500 ng/ml (LPS-500), or 1000 ng/ml (LPS-1000) of LPS. Bone marrow (BM) cDNA from C57BL/6 mice was used as a positive control and revealed the expected 264 bp product. *Lower panel*: corresponding GAPDH controls. The + RT lanes are shown adjacent to their corresponding - RT controls. M = DNA ladder; NTC = no template control.

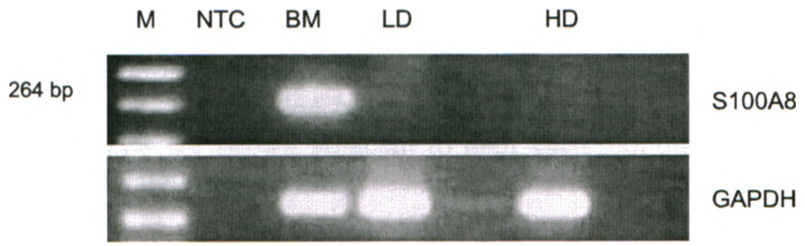

Fig. 3-2. RT-PCR analysis of S100A8 expression in P388D1 cells at different densities treated with LPS. *Upper panel*: P388D cells plated at low (LD; 40-50% confluence) and high (HD; 95-100% confluence) densities showed no expression of S100A8 mRNA following stimulation with 10 ng/ml of LPS for 20 hrs followed by a challenge dose for an additional 20 hrs with 500 ng/ml. Bone marrow (BM) cDNA from C57BL/6 mice was used as a positive control and revealed the expected 264 bp product. *Lower panel*: corresponding GAPDH controls. The + RT lanes are shown adjacent to their corresponding - RT controls. M = DNA ladder; NTC = no template control.

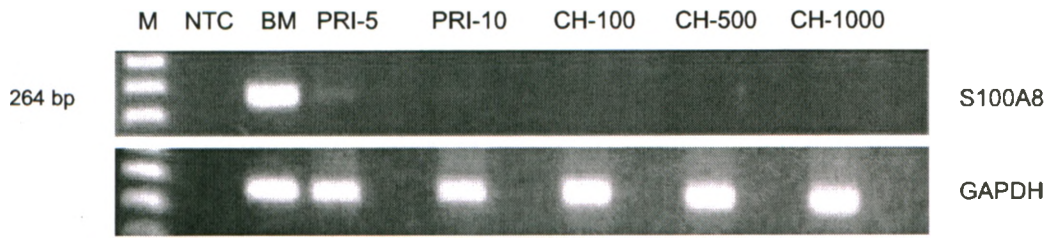

Fig. 3-3. RT-PCR analysis of S100A8 expression in LPS-primed or challenged P388D1 cells. *Upper panel*: P388D cells showed no expression of S100A8 mRNA with priming only at 5 ng/ml (PRI-5) or 10 ng/ml (PRI-10) of LPS for 20 hrs, or when challenged only at 100 ng/ml (CH-100), 500 ng/ml (CH-500), or 1000 ng/ml (CH-1000) of LPS for 20 hrs. Bone marrow (BM) cDNA from C57BL/6 mice was used as a positive control and revealed the expected 264 bp product. *Lower panel*: corresponding GAPDH controls. The + RT lanes are shown adjacent to their corresponding - RT controls. M = DNA ladder; NTC = no template control.

3.2 ATTEMPTS TO INDUCE S100A8 PROTEIN IN 32D CELLS

To be able to utilize the 32D cell line for the knockdown experiments, it was first necessary to show that induction of S100A8 with GCSF was possible in these cells. Expression of S100A8 and S100A9 was analyzed in 32D cells treated with 60 pg/ml of GCSF for 12, 24 and 48 hrs (Fig. 3-4), as well as cells subjected to different doses of GCSF (20, 60, 100, 250, 500 or 1000 pg/ml) for a period of 48 hrs (Fig. 3-5). After a number of trials, however, Western blotting failed to reveal S100A8 or S100A9 protein in GCSF-stimulated 32D cells. A major difficulty encountered in these experiments was culturing these cells in the absence of IL-3, which caused the majority of the cells to die after only 1-2 days of growth. Nacken *et al.* (2000) indicated that they were able to grow these cells for up to two weeks in the absence of IL-3, although cell death was evident after 2-3 days upon GCSF treatment (personal correspondence). Their study spanned a period of eight days, during which the expression of S100A9 (and S100A8) increased proportionally. In contrast, it was not possible to analyze induction of the two proteins beyond two days in this study.

32D cells were treated with a combination of GCSF and IL-3 to see if the expression of the two S100 proteins can be induced by permitting the cells to survive longer in culture. Again, no S100A8 protein was detected (Fig. 3-6). Surprisingly, in one treatment, in which the cells were sustained in culture for six days on a combination of 60 pg/ml GCSF and 100 pg/ml IL-3, a band was seen at approximately 13 kDa, representative of S100A9 (Fig. 3-7). These results could not be reproduced since it was not possible to culture another batch of cells (with GCSF) for the same period of time.

Since release of S100A8 and S100A9 has been reported in neutrophils, the secretion of S100A8 by 32D cells was examined by Western blotting. However, analysis of the supernatant (media) of GCSF-treated cells did not reveal expression of S100A8 (data not shown). Difficulties were encountered in precipitating total protein from the culture media and re-dissolving the protein pellet in SDS buffer. A more effective method is required to precipitate the total protein in the future in order to reveal the levels of S100A8 protein, if any, in the supernatant of GCSF-stimulated 32D cells.

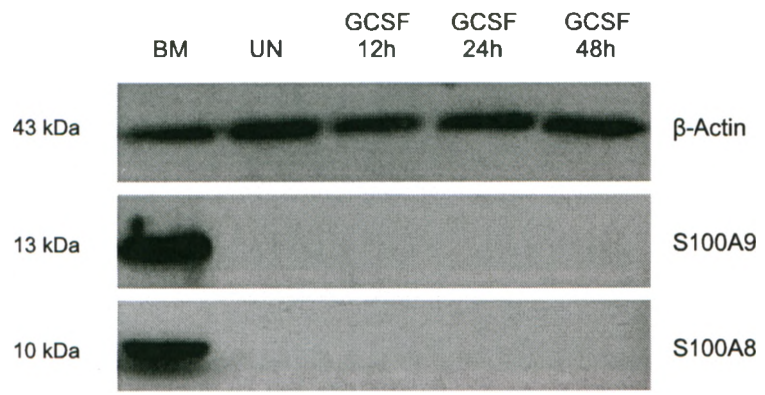

Fig. 3-4. Western blot analysis of S100A8 and S100A9 expression in 32D cells. Cells were treated with 60 pg/ml GCSF for 12, 24 and 48 hrs (lanes 3, 4, and 5, respectively). Untreated (UN) cells (maintained on 100 pg/ml IL-3 only) served as a negative control, and bone marrow (BM) total protein from C57BL/6 mice was used as a positive control. β-actin served as the loading control. Molecular masses (kDa) are indicated on the left.

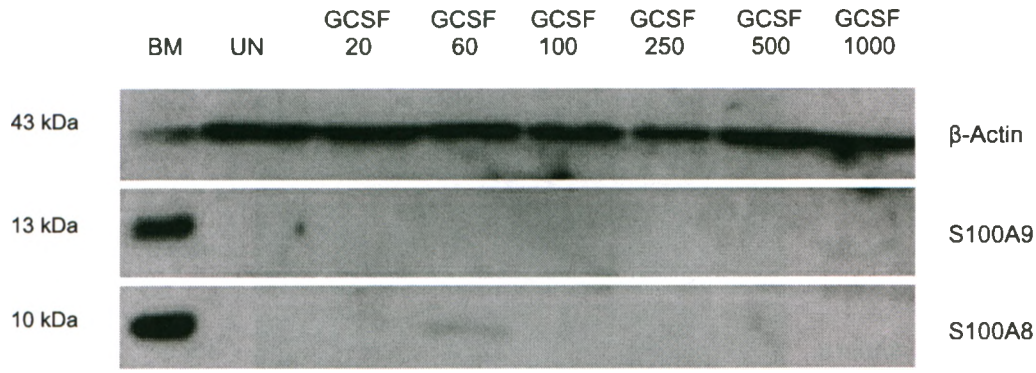

Fig. 3-5. Western blot analysis of S100A8 and S100A9 expression in GCSF-treated 32D cells. Cells were treated with various concentrations of GCSF (20, 60, 100, 250, 500, or 1000 pg/ml) for a period of 48 hrs. Untreated cells (maintained on 100 pg/ml IL-3 only) served as a negative control (lane 2), and bone marrow (BM) total protein from C57BL/6 mice was used as a positive control. β-actin served as the loading control. Molecular masses (kDa) are indicated on the left.

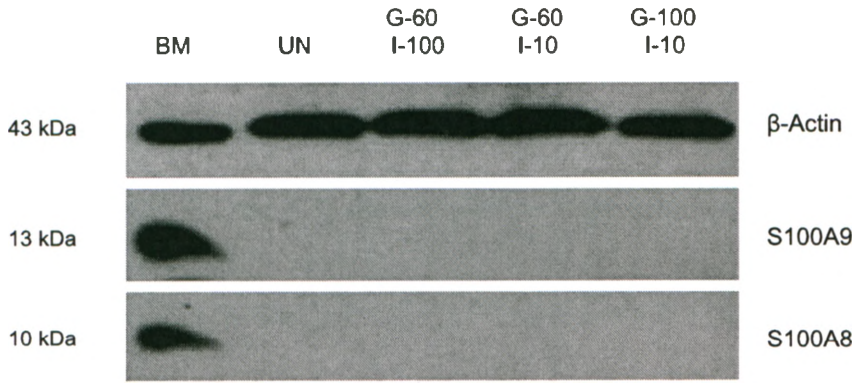

Fig. 3-6. Western blot analysis of S100A8 and S100A9 expression in 32D cells. Cells were treated with various combinations of GCSF and IL-3 for a period of 48 hrs. Lane 3, treatment with 60 pg/ml GCSF + 100 pg/ml IL-3; lane 4, treatment with 60 pg/ml GCSF + 10 pg/ml IL-3; lane 5, treatment with 100 pg/ml GCSF + 10 pg/ml IL-3. Untreated cells (maintained on 100 pg/ml IL-3 only) served as a negative control (lane 2), and bone marrow (BM) total protein from C57BL/6 mice was used as a positive control. β-actin was used a loading control. Molecular masses (kDa) are indicated on the left.

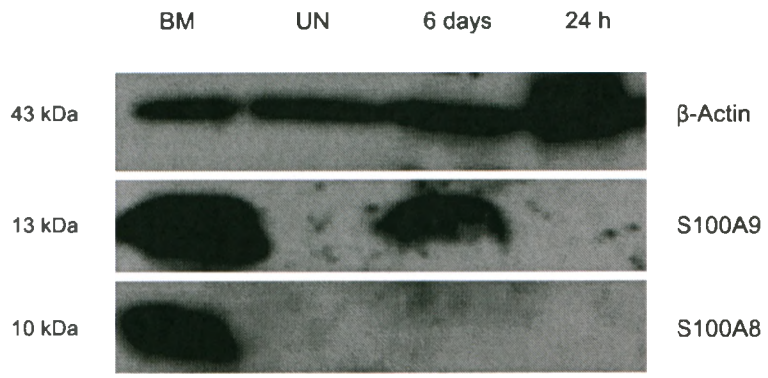

Fig. 3-7. Western blot analysis of S100A8 and S100A9 expression in 32D cells. Cells were treated with 60 pg/ml GCSF + 100 pg/ml IL-3 for 24 hrs (lane 4) or six days (lane 3). Untreated cells (maintained on 100 pg/ml IL-3 only) served as a negative control (lane 2), and bone marrow (BM) total protein from C57BL/6 mice was used as a positive control. β-actin was used a loading control. Molecular masses (kDa) are indicated on the left.

3.3 GENERATING STABLE S100A8 CELL LINES

3.3.1 Analysis of S1008 Expression in Stable Clones

COS-7 cells were transfected with the pDream2.1 S100A8-expressing plasmid, and grown for six weeks under G418 selection until stable clones were obtained. Three independent clones (C1, C2 and C3) showed positive expression of S100A8 by RT-PCR (Fig. 3-8).

3.3.2 S100A8 Protein Analysis in Stable Clones

Total protein from COS-7 cells transfected transiently and stably with S100A8 (Cl, C2 and C3) was collected and analyzed for the presence of S100A8 protein by Western blotting. No S100A8 protein was detected in any of the three stable clones, although numerous attempts were made (Fig. 3-9). Manipulating the amount of total protein loaded onto the gel (50-100 μg) as well as other experimental conditions did not reveal S100A8 protein expression in the stable COS-7 cell lines.

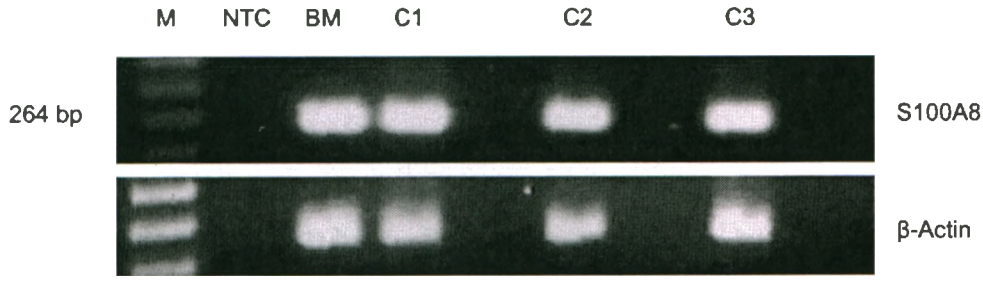

Fig. 3-8. RT-PCR analysis of S100A8 expression in stably transfected COS-7 cells. *Upper panel*: three independent clones of COS-7 cells (C1, C2 and C3) stably transfected with S100A8 plasmid show expression of S100A8 (264 bp product). Bone marrow (BM) cDNA from C57BL/6 mice was used as a positive control. *Lower panel*: corresponding β-actin controls. The + RT lanes are shown adjacent to their corresponding - RT controls. M = DNA ladder; NTC = no template control (lane 1).

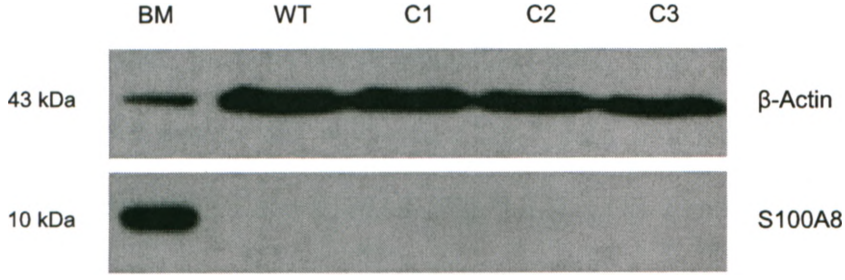

Fig. 3-9. Western blot analysis of S100A8 expression in stable COS-7 cells. Lane 2 represents non-transfected wild-type (WT) control cells. Lanes 3, 4, and 5 represent lysates (50 μg) from cells stably transfected with murine S100A8 (clones, C1, C2 and C3). The expected 10 kDa S100A8 protein band is absent. Bone marrow (BM) total protein from C57BL/6 mice was used as a positive control. β-actin served as the loading control. Molecular masses (kDa) are indicated on the left.

3.4 GENERATING STABLE S100A8/S100A9 CELL LINES

Since S100A8 protein could not be detected by Western blotting, and to test the theory that S100A8 protein requires S100A9 for stability, murine S100A9 was introduced into the stable cells to determine the effect on S100A8 protein levels. COS-7 cells stable for S100A8 expression transfected transiently with S100A9 showed S100A9 protein at 24 and 72 hrs post-transfection but no S100A8 protein was detected in either sample (Fig. 3-10). Furthermore, wild-type COS-7 cells transfected transiently with S100A8, S100A9, or both S100A8 and S100A9 showed S100A9 protein exclusively (Fig. 3-11). Stable S100A8/S100A9 clones were generated with hygromycin selection at 100 μg/ml. Again, these clones showed S100A9 protein only (Fig. 3-12, 3-13).

To test the possibility that the S100A8 protein might be degraded by proteosomes, another transient transfection experiment was performed in which cells were treated with either 10 μM MG-132 for 4 hrs or 50 μM MG-132 for 2 hrs (cell lysates were collected 48 hrs post-transfection). MG-132 is an inhibitor of proteasomes and reduces degradation of ubiquitin-conjugated proteins in mammalian cells. This experiment revealed expression of S100A9 protein for wild-type COS-7 cells transfected transiently with S100A9 only or both S100A8 and S100A9 (Fig. 3-14). COS-7 cells stable for S100A8 expression transfected transiently with S100A9 also revealed S100A9 protein but no S100A8. Thus, treatment with MG-132 failed to reveal S100A8 protein.

The two S100 proteins are known to be secreted by certain cell types, including human monocytes and stimulated granulocytes. Thus, media collected from cells stably transfected with S100A8 or both S100A8 and S100A9 was analyzed. However, S100A8 or S100A9 protein was not detected the media of these cells (data not shown).

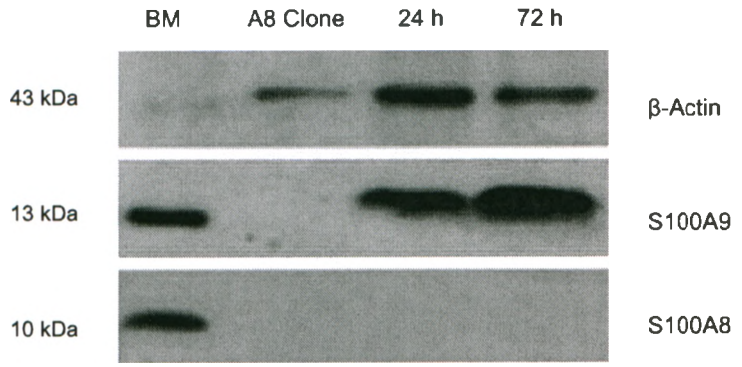

Fig. 3-10. Western blot analysis of S100A8 and S100A9 expression in COS-7 cells. Cells stable for S100A8 were re-transfected (transiently) with S100A9. Cell lysates collected 24 and 72 hrs post-transfection (lanes 3 and 4, respectively) show anti-S100A9 immunoreactivity at 13 kDa but no S100A8 protein is detected. Cells stable for S100A8 expression (A8 Clone, untransfected with S100A9) served as a negative control, and bone marrow (BM) total protein from C57BL/6 mice was used as a positive control. β-actin served as the loading control. Molecular masses (kDa) are indicated on the left.

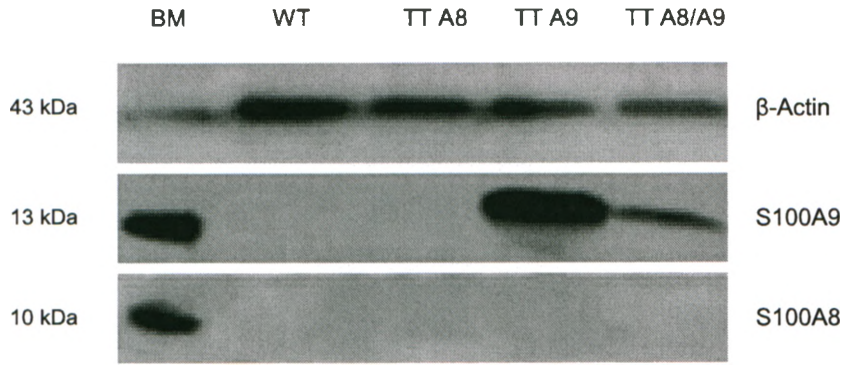

Fig. 3-11. Western blot analysis of S100A8 and S100A9 expression in transiently transfected COS-7 cells. Wild-type COS-7 cells were transfected transiently (TT) with S100A8 (lane 3), S100A9 (lane 4), or both S100A8 and S100A9 (lane 5). Both transfections involving S100A9 revealed the expected S100A9 product (13 kDa) but no S100A8 protein is present. Wild-type (WT) COS-7 cells (untransfected) served as a negative control, and bone marrow (BM) total protein from C57BL/6 mice was used as a positive control. β-actin served as the loading control. Molecular masses (kDa) are indicated on the left.

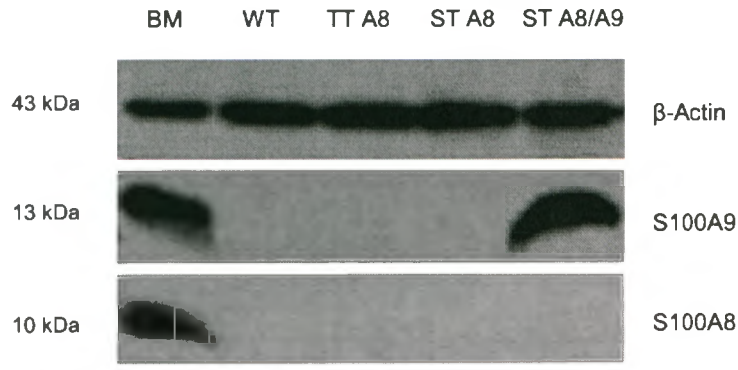

Fig. 3-12. Western blot analysis of S100A8 and S100A9 expression in COS-7 cells. Cells transiently transfected (TT) (lane 3) and stably transfected (ST) (lane 4) for S100A8 expression did not show S100A8 protein. Cells stable for both S100A8 and S100A9 (lane 5) reveal the expected S100A9 protein product (13 kDa) but no S100A8 protein. Wild-type (WT) COS-7 cells (untransfected) served as a negative control, and bone marrow (BM) total protein from C57BL/6 mice was used as a positive control. β-actin served as the loading control. Molecular masses (kDa) are indicated on the left.

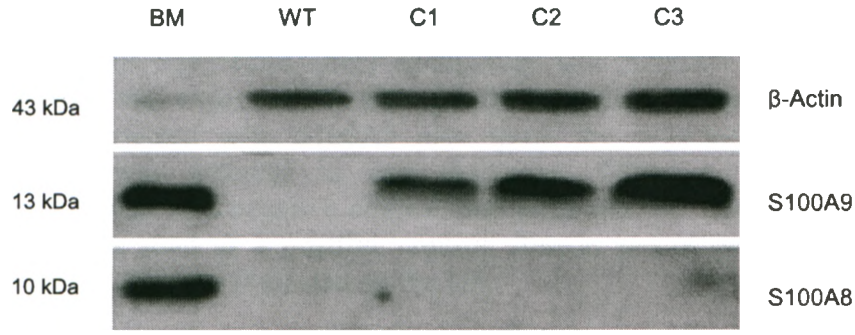

Fig. 3-13. Western blot analysis of S100A8 and S100A9 expression in stable S100A8/S100A9 clones. Lane 2 represents non-transfected wild-type (WT) control cells. Lanes 3, 4, and 5 represent lysates from cells stably transfected with S100A8 re-tranfected (stably) with S100A9 (clones, C1-3). The expected 13 kDa S100A9 protein band is evident in the stable clones. Bone marrow (BM) total protein from C57BL/6 mice was used as a positive control. β-actin served as the loading control. Molecular masses (kDa) are indicated on the left.

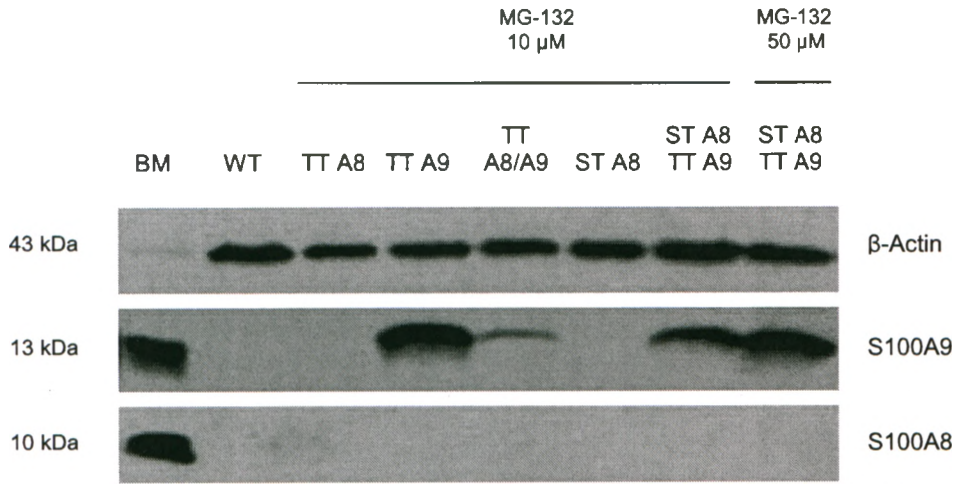

Fig. 3-14. Western blot analysis of S100A8 and S100A9 expression in MG-132-treated COS-7 cells. Wild-type and cells stable for S100A8 were transfected (transiently) with S100A9. Cell lysates were collected 48 hrs post-transfection, following treatment with two different regimes of MG-132 (either 10 μM for 4 hrs or 50 μM for 2 hrs). Wild-type cells transfected either exclusively with S100A9 or both S100A8 and S100A9 revealed the expected 13 kDa S100A9 product. Cells stable for S100A8 expression re-transfected with S100A9 also show the expected S100A9 band. No S100A8 protein was detected in any treatment. Wild-type (WT) COS-7 cells (untransfected) served as a negative control, and bone marrow (BM) total protein from C57BL/6 mice was used as a positive control. β-actin served as the loading control. Molecular masses (kDa) are indicated on the left.

3.5 KNOCKDOWN OF S100A8 WITH SHORT-HAIRPIN RNA

3.5.1 Sequence Alignment of Mouse S100 Genes

To verify that the shRNAs against S100A8 obtained for this study will not target other murine S100 genes, a sequence alignment of all known mouse S100 genes was performed. It was evident from the results of the sequence alignment that the target sequences of the shRNAs are specific for S100A8 (Fig. 3-15).

S100A4	GGC-CCTGGATGTAATTGTGTCCA	95	AGGAGTACTGTGTCTTCCTGTCC	292	CAGTGGGGGATCTTCCCTG-----	412
S100A7	CTC-CCTCTTCCAAATCATACACT	156	ATGAGTTCCTGTACATCTTGGGC	359	CACCAGAACCTCTCCCGAGACATG	484
S100A10	CGC-CATGGAAACCATGATGCTTA	158	AGAGCTTTCTATCACTAGTGGCA	346	GTGCGTGTTCACCCACGGGGTC--	461
S100Z	GGC-TATGGACACCATGATTCGCA	43	ATGAGTTTGTGGTCATGGTGGCA	240	----------	
S100A1	TGC-CATGGAGACCCTCATCAATG	186	AGGAGTATGTTGTGCTGGTGGCT	383	TAGCGTGCTTGCTCCTCACCCC--	502
S100B	GGC-CATGGTTGCCCTCATTGATG	171	AGGAGTTCATGGCCTTCGTCGCC	368	GAGGAGGCACAGCAAAGGCTCATG	502
S100A9	CAG-CATAACCACCATCATCGACA	88	AGGAGTGTATGATGCTGATGGCA	288	AAGAGGTCAGC-CATGTGACAGCT	400
S100A11	ATG-CATTGAGTCCCTGATTGCTG	109	AAGAGTTTCTCAACCTCATTGGT	306	AAGTCATCACCTCCCCCGACCCCA	412
S100A5	GGC-ACTGACCACCATGGTCACCA	167	AGGAGTACTCTGTGTTCCTGACC	258	CTTTAACTGCCC--CTCCTATGGC	368
S100A6	GGCCATTGGCCTTC-TCGTGGCCA	400	AGGAGTATGTCGCCTTCCTGGGG	591	-GGTGGGTAGTT--ATACAATAAA	598
S100A3	GGC-AGTAGCTGCCATCGTGTGCA	102	GGGAGTACGTGCGCTCACTTGCC	299	TGTACGCTATCCCAGAAGGGCAG-	403
S100A16	GGC-AGTTGTTGTCCTGGTGGAAA	173	ACGAATACTGGACCATGATTGGT	373	CAGCAGGAGAGCCAGCAGGGCAGC	490
S100A8	GGC-CTTGAGCAACCTCATTGATG	98	AGGAGTTCCTTGCGATGGTGATA	283	CCCTGGATATGT-CTACAGAATAA	373
S100G	GTC-TCCTGCAGAAATGAAGAGCA	133	AAGAATTCGAAGCTTTCTTCAAA	321	CTCTAGCTGTGCGATTATGATACA	425
S100A13	CGGTCCCCTCAGCGGAGGCGGAAA	158	GTCTCTACTTTCTTCACCTTTGC	371	ATGAAGACCTTGGATGTGAATCAG	501
S100A14	GGC-CATTGAGACACTCATCAAGA	204	AAGCAGCCAAGAGTGTGAAGATG	413	CCACCACCCCCTTCCCCAGCCTGC	538

Fig. 3-15. Sequence alignment of mouse S100 genes, showing target sequences of shRNA_mA8_65 (red), shRNA_mA8_250 (blue), and shRNA_mA8_340 (green) on murine S100A8 cDNA.

3.5.2 Establishing Stable shRNA Cell Lines

Transient transfections of shRNAs into the COS-7 cells stably expressing S100A8 exhibited very poor efficiency as shown by the scarcity of fluorescent cells (Fig. 3-16A-B). Experimental conditions were manipulated in various ways by using different transfection reagents (including Superfectamine™), varying the density of transfected cells, altering the lipid-to-DNA ratio and by employing different transfection protocols. The shRNA plasmids were also re-purified and transient transfections were attempted again, but the efficiency of transfection could not be improved. Toxicity and cell death presented an additional problem during these transient transfection experiments.

Therefore, cell lines expressing shRNAs stably in COS-7 cells already transfected with S100A8 were generated. This system would provide stable, long-term expression of the shRNA of interest. Cell lines for each of the vectors expressing shRNA_mA8_65, shRNA_mA8_250, shRNA_mA8_340 and the non-relevant shRNA_CLCA3_665 (control) were produced. Fluorescence microscopy confirmed delivery of the shRNAs because untransfected cells were not fluorescent (Fig. 3-16A) compared to transfected cells (Fig. 3-16C-F). Visual observation of fluorescence intensities of cells transfected stably with shRNA suggests that the cell lines were similar in terms of GFP expression. The intensity of fluorescence in individual cells varied, however, with intense fluorescence in the cytoplasm of some cells compared to other cells.

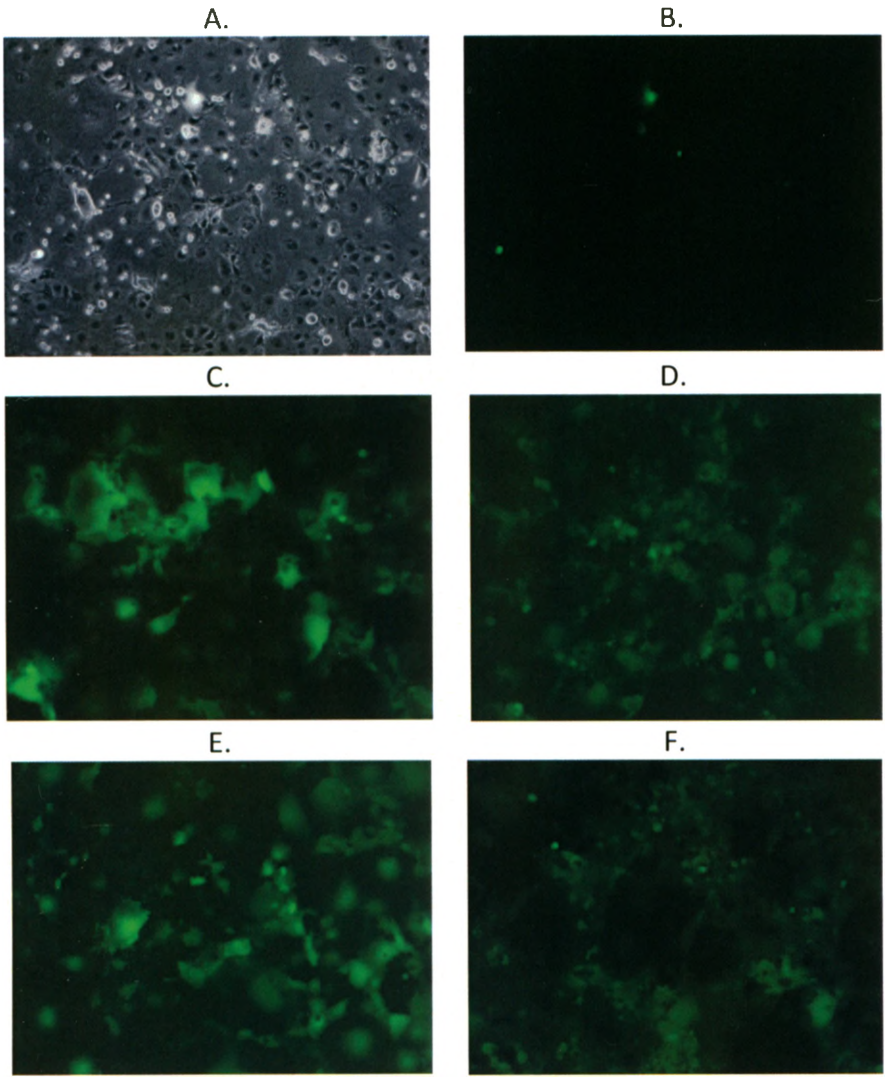

Fig. 3-16. GFP expression in COS-7 cells. Cells were **(A)** untransfected or **(B)** transfected transiently with shRNA_mA8_65 (image taken 48 hrs post-transfection), or transfected stably with **(C)** shRNA_mA8_65, **(D)** shRNA_mA8_250, **(E)** shRNA_mA8_340 and **(F)** non-relevant (control) shRNA. Transient transfections revealed that very few cells exhibited fluorescence (B) when compared to the corresponding field of view in (A). Fluorescence images of stably transfected cell lines confirmed that all cells were expressing GFP (Magnification: x100).

3.5.3 S100A8 Expression in shRNA-transfected Cells Lines

Single colonies of stably-transfected COS-7 cells were selected by serial dilution to generate homogenous cell lines and to compare levels of S100A8 expression in response to shRNA treatment. The average knockdown for each shRNA was determined by real-time RT-PCR. Expression of S100A8 (relative to β-actin) in COS-7 cells treated with shRNA_mA8_65 and shRNA_mA8_250 was significantly less (0.46 ± 0.05, P=0.001 and 0.90 ± 0.12, P=0.033, respectively) than that of the untreated control cells (1.21 ± 0.05), achieving 62% and 25% suppression of S100A8, respectively (Fig. 3-17). The third hairpin, shRNA_mA8_340, did not significantly reduce the levels of S100A8 (1.15 ± 0.10, P=0.600) compared to the untreated controls. Moreover, as expected, S100A8 expression in cells treated with the control shRNA vector was comparable to that of the untreated control cells (1.27 ± 0.14, P=0.686).

After checking the target sequence of shRNA_mA8_340, which failed to down-regulate S100A8 expression, it was discovered that the target sequence lies in the 3' UTR of the S100A8 gene (Fig. 2-4 in Materials & Methods), and therefore, is not expected to have any effect on S100A8 levels since only the 270 bp coding sequence of the murine S100A8 gene (bases 56 to 325) was transfected stably into the COS-7 cells. Nonetheless, shRNA_mA8_340 served as a negative control.

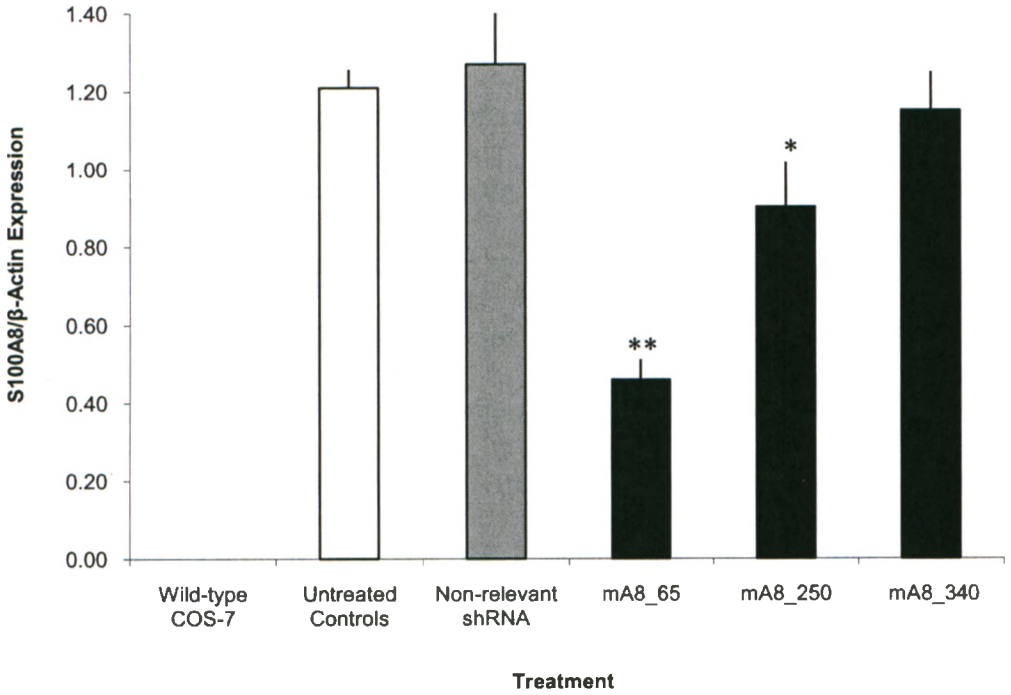

Fig. 3-17. S100A8 expression in stable shRNA cell lines. Real-time RT-PCR analysis of S100A8 expression (normalized to β-actin) in shRNA-treated and untreated (wild-type) COS-7 cells stable for S100A8 expression (performed in duplicates with N=6 per treatment group). The expression levels of S100A8 in cells treated with shRNA_mA8_65 and shRNA_mA8_250 (black bars) were significantly less (P=0.001 and P=0.033, respectively) than that for the untreated (white bar) and non-relevant controls (grey bar). shRNA_mA8_340 did not result in a significant decrease of S100A8 (P=0.600) compared to the untreated controls. Treatment with a control vector had no effect on the levels of S100A8 compared to the untreated controls. The wild-type cells show no basal levels of S100A8. Values represent means ± SEM. Asterisks represent significance (*P<0.05, **P<0.01).

3.5.4 GFP Expression in Stable shRNA Cell Lines

To confirm that the variation in the suppressive activity of the shRNAs was not due to differences in the rate of their transcription, it was important to assess their level of expression. However, due to the highly structured template of the hairpin and the complex secondary structures that are unique to each sequence, it would have been difficult, if not impossible, to derive primers to efficiently amplify the RNA transcripts of the hairpins for qRT-PCR analysis. Investigators often analyze the expression of a marker gene expressed from the same construct as the shRNA (GFP in this case), and correlate this to shRNA production levels (Dickins *et al.*, 2005; Taxman *et al.*, 2006). In the MSCV-LTRmiR30-PIG shRNA expression vectors, GFP is driven primarily by the 5' LTR, and, to a lesser degree, by a PGK promoter, which drives the expression of the puromycin resistance gene. However, the PGK promoter is not strong enough to drive GFP expression at high enough levels for detection. In the vector, GFP lies downstream of an IRES sequence, such that when LTR drives expression of the shRNAs it runs through the PGK promoter and puromycin resistance gene and includes GFP on the same transcript. Thus, the predominant transcript from this vector is driven by LTR.

Accordingly, GFP expression was analyzed by real-time RT-PCR. GFP expression (relative to β-actin) was found to be comparable for all four treatment groups with no significant differences between the control shRNA (1.04 ± 0.08), shRNA_mA8_65 (1.12 ± 0.09), shRNA_mA8_250 (1.08 ± 0.13), or shRNA_mA8_340 (1.06 ± 0.16) with $P>0.05$ (Fig. 3-18). The untreated (control) cells showed no expression of GFP, as expected. This confirms that the level of S100A8 knockdown achieved by each shRNA is representative of the shRNA's efficacy and potency.

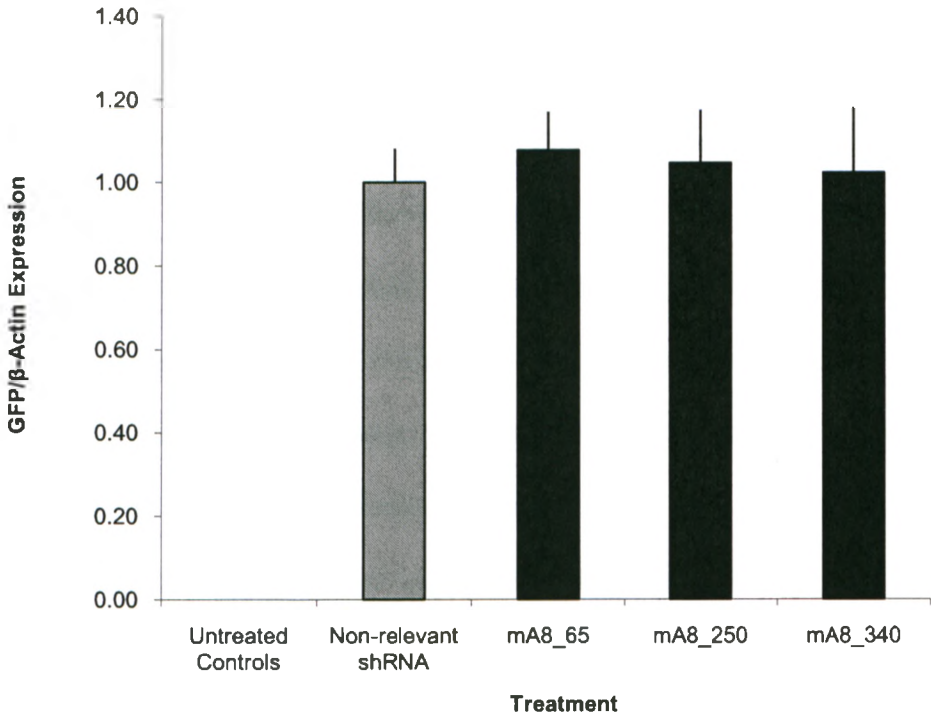

Fig. 3-18. GFP expression in stable shRNA cell lines. Real-time RT-PCR analysis of GFP mRNA expression (normalized to β-actin) in untreated COS-7 cells or cell transfected stably with non-relevant control shRNA (grey bar), shRNA_mA8_65, shRNA_mA8_250, shRNA_mA8_340 (black bars) (performed in duplicate with N=4 per treatment group). There are no significant differences (P>0.05) in GFP expression between the four shRNA treatments. Untreated (control) cells showed no expression of GFP, as expected. Values represent means ± SEM.

4. DISCUSSION

4.1 ANALYSIS OF S100A8 EXPRESSION IN P388D CELLS

4.1.1 Expression of S100A8 in Unstimulated P388D1 Cells

The expression of S100A8 and S100A9 is constitutive in neutrophils; however, their expression in other myeloid cells is dependent on the differentiation state of the cell (Roth *et al.*, 1993b; Teigelkamp *et al.*, 1991; Zwaldo *et al.*, 1986). In the study by Goebeler *et al.*, (1993), the authors investigated S100A8/S100A9 expression in various murine myelomonocytic cell lines, including M1, RMB.TG, WEHI.TG, J774A and P388D1. Protein expression in these cells was assessed by qualitative examination of immunoperoxidase-stained cytospin preparations. Each of these cell lines represented a distinct stage of monocyte differentiation, with P388D1 cells being the furthest down the path of maturation (Goebeler *et al.*, 1993). The more immature cell lines, M1 and RMB.TG, did not stain for S100A8/S100A9, while WEHI.TG cells stained weakly, and J774A and P388D1 cells revealed weak to moderate levels of S100A8/S100A9 (staining in comparison to granulocytes from peripheral blood smears) (Goebeler *et al.*, 1993). The authors concluded that S100A8/S100A9 expression is associated with an intermediate stage of macrophage differentiation (Goebeler *et al.*, 1993).

In this study, however, it was not possible to detect the expression of S100A8 in P388D1 cells using RT-PCR. Assessment of S100A8 expression has proven to be a difficult task as reported by several authors working extensively with this protein (Geczy, personal correspondence; Kerkhoff, personal correspondence). In general, previously published results are not always reproducible owing to differences in protocols, media components and culturing conditions which can alter the physiological state of the cells

in the study. For instance, S100A8 expression reported in murine T79 skin cancer cells (Gebhardt *et al.*, 2006) could not be verified by Geczy *et al.* when they investigated this cell line for constitutive S100A8 mRNA expression (personal correspondence).

4.1.2 Attempts to Induce S100A8 Expression with LPS

Monocytes have been shown to express S100A8 and S100A9 at low levels, whereas macrophages normally do not express these proteins (Goebeler *et al.*, 1993; Hsu *et al.*, 2005). It is possible, however, to induce S100A8 expression by certain pro-inflammatory mediators. For example, LPS, IFN-γ, IL-1β and TNF-α have been shown to up-regulate S100A8 expression in human monocytes (Hsu *et al.*, 2005; Kido *et al.*, 2005; Suryono *et al.*, 2003; Zwaldo *et al.*, 1986). In murine systems, S100A8 expression is up-regulated in macrophages/monocytes with LPS, TNF-α and IFN-γ (Hsu *et al.*, 2005; Hu *et al.*, 1996; Xu *et al.*, 2001; Xu and Geczy, 2000), as well as in murine NIH3T3 fibroblast cells treated with LPS, IL-1β or FGF-2 (Rahimi and Geczy, 2005).

In this study, however, S100A8 expression in P388D1 cells was not induced following stimulation with different priming and challenge doses of LPS. This may suggest that the state of differentiation of P388D1 macrophages may be a factor in determining their response to LPS, as reported by Hu *et al.* (1996). In the study by Hu *et al.* (1996), bone marrow-derived macrophages did not reveal expression of S100A8 following priming and secondary challenge with LPS, and it was suggested that the immature stage of the cells was responsible for the lack of S100A8 expression. P388D1 cells are mature macrophages, as indicated by their high expression of mature macrophage markers such as Mac-1 and F4/80 (Goebeler *et al.*, 1993; Leenan *et al.*, 1986). Thus, the mature differentiation state of the P388D1 cells used in this study could

be responsible for the lost ability of these cells to express S100A8, under both normal and stimulated conditions, as part of their differentiation to maturity. Indeed, the maturation of monocyte precursors has been shown to coincide with the down-regulation of S100A8 and S100A9 (Goebeler *et al.*, 1993; Lagasse and Clerc, 1988; Zwaldo *et al.*, 1988), reflecting different functional properties of these cells (Roth *et al.*, 1993b).

It is also worth noting that P388D1 cells represent a tumor cell line to which macrophage properties have been attributed (Koren *et al.*, 1975). So in essence, these cells may not possess the capacity to respond to LPS as do other true macrophage cells. It may also be that the LPS concentrations used and/or duration of treatments were not optimal to induce the expression of S100A8 in this cell line; though the conditions used were based on studies involving other murine macrophage cells. Furthermore, the degree of confluency may be an important factor in the induction of S100A8 (Rahimi *et al.*, 2005; Yen *et al.*, 1997). In one study, it was found that at higher densities of RAW 264.7 cells, there was an enhanced LPS-activated IFN-β signaling pathway, which was shown to augment S100A8 induction in macrophages (Jacobs and Ignarro, 2003). In another study, cell density affected the induction intensity of S100A8 mRNA in NIH3T3 cells (Rahimi *et al.*, 2005). In the present study, however, varying the concentration of P388D1 cells treated with LPS failed to reveal S100A8 mRNA transcripts.

Although LPS has been shown to be a strong inducer of S100A8 in macrophages, the precise mechanisms are still not fully understood due to the complex nature of the signaling cascade and the multiple effects of LPS on these cells. The attempt to induce S100A8 expression in P388D1 cells with LPS has not been reported previously, and it was presumed that S100A8 levels could be up-regulated upon treatment with LPS. Thus,

it was decided that P388D1 cells may not be suitable for assessing S100A8 knockdown. Induction of S100A8 expression in other murine cell lines, such as RAW 264.7, may have provided better results, since these cells have been used extensively to investigate S100A8 gene regulation. Moreover, S100A8 is induced in the absence of S100A9 in RAW 264.7 cells (Hsu *et al.*, 2005; Xu *et al.*, 2001), which potentially makes this a valuable system for assessing the role of S100A8 in future studies.

4.2 INVESTIGATION OF S100A8 EXPRESSION IN 32D CELLS

GCSF is a growth factor produced by a number of different tissues to stimulate the bone marrow to produce granulocytes and stem cells (Souza *et al.*, 1986). In this study, GCSF was used in an effort to stimulate S100A8 expression in murine 32D cells. The idea was to generate stable shRNA-transfected 32D cells in the undifferentiated state, followed by treatment with GCSF to induce differentiation into the granulocytic lineage and induction of S100A8. However, it was not possible to reproduce the work of Nacken *et al.* (2000). In their study, it was demonstrated that GCSF-mediated differentiation in 32D cells up-regulated S100A9 (as well as S100A8) protein levels, which was also detected in the supernatant (Nacken *et al.*, 2000). Other studies have also demonstrated induction of S100A8 expression upon differentiation along the granulocytic lineage (Collins *et al.*, 1979; Lagasse and Clerk, 1988; Roth *et al.*, 1993b; Warner-Bartnicki *et al.*, 1993). The present study, however, failed to detect S100A8 protein in 32D cells following treatment with different concentrations of GCSF.

One problem encountered in this study was maintaining 32D cells in culture in the absence of IL-3, which is required for their proper proliferation. This is in accordance with the fact that GCSF results in differentiation of granulocyte precursors to neutrophil-

like cells, which are short-lived. Only one treatment was sustained for six days and revealed S100A9, but no S100A8, protein. This, however, was not validated further because cells could not be cultured for long in media deficient in IL-3, or, protein concentration and quality was very poor when cells were maintained in culture longer than two days. If confirmed, this finding may reflect the fact that murine S100A8 and S100A9 are not dependent on each other for stability, since S100A9 appeared to be expressed exclusively in the absence of detectable levels of S100A8 protein.

It is not exactly clear why the cells could not be cultured in the absence of IL-3 for longer periods or why the cells did not respond to stimulation by GCSF to produce S100A8 protein during the initial 1-2 days of treatment. Differences in the potency of the GCSF used in this study compared to that used by Nacken *et al.* (2000) may have played a role in the results observed here; though this possibility was assessed by testing different concentrations of GCSF. Induction of apoptosis has been shown to cause rapid and substantial inhibition of protein synthesis (Marissen and Lloyd, 1998), which accounts for the low levels and poor quality of protein collected from GCSF-treated cells.

It is important to note that a cell system in which S100A8 expression is induced and suppressed simultaneously may not be suitable for RNAi experiments as this may interfere with gene silencing. Endoh *et al.* (2009) outlined the difficulties they experienced in suppressing S100A8 in cells in which S100A8 expression needed to be induced concurrently. They were unsuccessful in down-regulating LPS-mediated S100A8 induction in RAW 264.7 cells with siRNAs, which actually enhanced S100A8 levels (Endoh *et al.*, 2009). This may have been due to an imbalance between mRNA degradation by the siRNA and induction by stimulants (Endoh *et al.*, 2009). Moreover,

the suppression pathway may become saturated, such that at low expression levels, the RNAi machinery may be able to degrade most target mRNAs, whereas a strongly-induced gene will produce excessive mRNA transcripts that may not be processed adequately by RISC (Bitko and Barik, 2001; Hutvagner *et al.*, 2004), leading to an apparent insufficiency of the shRNA. It was concluded, therefore, that an alternative approach is required to show S100A8 expression and subsequent inhibition with shRNA.

4.3 S100A8 EXPRESSION IN STABLE CELL LINES

At the time of this study, there were no reports of the successful generation of a cell line over-expressing the murine S100A8 gene; thus, stable COS-7 cell lines expressing S100A8 were generated. S100A8 mRNA expression was verified in the stable clones but no S100A8 protein was detected. In mouse trophoblasts, S100A8 mRNA accumulates to high levels without a corresponding increase in S100A8 protein, which the authors suggested was actively secreted (Passey *et al.*, 1999a). However, Western blot experiments also failed to detect S100A8 protein the supernatant of the stable cells, indicating that the protein is either absent or at least not detectable with the antibody used here, although the bone marrow positive control revealed S100A8 protein.

The problem of S100A8 protein detection has been reported by several authors, including Geczy *et al.* who indicated that they also experienced problems in showing S100A8 protein in transfected cell lines (personal correspondence). For some genes expressed in certain systems, the mRNA can go through a silencing process and large amounts of mRNA produced do not necessarily translate into large amounts of protein. There may be biological causes for this lack of correlation between mRNA and protein levels, including varying protein stability and post-translational modifications. For

example, in S100A9 gene silencing experiments, Kerkhoff *et al.* (2005) failed to detect S100A8 protein although S100A8-specific mRNA was present, suggesting that S100A8 protein is unstable in the absence of its interacting partner, S100A9. This was also consistent with two other reports of S100A9 deficient mice (Hobbs *et al.*, 2003; Mantiz *et al.*, 2003). Thus, since the stable COS-7 cells in this study were designed to express S100A8 exclusively, and due to the conflicting reports about the dimerization of S100A8 and S100A9 in the murine and human systems, the reasoning that the S100A8 homodimer is degraded in the cell in the absence of S100A9 seemed plausible.

Alternatively, inherent (technological) flaws within the gene and/or expression cassette may account for the differences in mRNA and protein levels. For instance, an inappropriate translational start site may reduce the efficiency of translation, or inhibit it altogether in some instances. Moreover, the presence of a stop codon within the gene sequence or a frame-shift will result in a truncated peptide that would eventually be degraded in the cell. The S100A8 gene cloned into pDream2.1 was sequenced to confirm the presence of a proper translational start and that no errors existed within the sequence. However, the remote possibility that a mutation may have arisen within the expression cassette in the stable cells following transfection and integration into the genome still exists, which may account for the lack of detectable levels of S100A8 protein.

Inefficient translation of S100A8 mRNA is a likely possibility that may explain the lack of protein in stable cells. The initiation of protein synthesis involves complex interactions between ribosomes, RNA molecules and a large number of initiation factors (Pain *et al.*, 1996). Efficient translation depends not only on the huge number of cellular components that provide regulatory control over the process, but also on structural

features and regulatory sequences within the mRNA template, including secondary or tertiary RNA structures, such as hairpins, which can block translation (Pain *et al.*, 1996). Moreover, S100A8 may not fold properly or its homodimer form may not be assembled correctly by the COS-7 machinery. Consequently, antibodies against S100A8 may not recognize the improperly folded protein. Thus, translational and/or post-translational limitations may have been the major bottlenecks for successful S100A8 expression.

Other possibilities exist for the presence of detectable levels of S100A8 mRNA without corresponding levels of S100A8 protein. There is a chance that the stable cells integrated only a few copies of the plasmid into their genome, such that there is sufficient expression of the antibiotic resistance gene to permit survival in selection media but not enough to produce S100A8 protein quantities detectable by Western blotting. Although as much as 100 μg of total protein from lysates of stably-transfected COS-7 cells was loaded, S100A8 protein was not seen. A more sensitive technique, such as an enzyme-linked immunosorbent assay (ELISA), may have provided better results.

Protein expression levels are generally hard to predict and are influenced to a great extent by the nature of the gene and its product (Carpentier *et al.*, 2007). For instance, the murine S100A8 gene product may be toxic to COS-7 cells. Consequently, the cell will attempt to shut down the production of the protein to salvage its integrity. As such, only the cells that are able to sequester and degrade the S100A8 protein proliferate (provided that they continue to express the neomycin resistance gene under selection conditions). Other researchers who attempted to create stable cell lines over-expressing murine S100A8 were unsuccessful, and they also suggest that this is possibly due to the potential cytotoxicity of the over-expressed protein (Geczy, personal correspondence).

The many complicated post-transcriptional processes involved in translating mRNA into protein are not yet fully understood to be able to compute protein levels from mRNA (Cho *et al.*, 1998). Whatever the case may be, it is clear that there are cellular limitations to protein over-expression and further work is necessary to identify and better understand these barriers. For this study, analysis of mRNA levels using real-time RT-PCR was deemed adequate to show S100A8 down-regulation following treatment with shRNAs. For future *in vivo* studies, however, determination of protein levels will be necessary to show the consequences of the reduction of S100A8 protein on the lung phenotype of CF mice, since, *in vivo*, it is the protein product of the S100A8 gene that is implicated in the inflammatory pathology of CF lungs.

4.4 INTERDEPENDENCE OF S100A8 AND S100A9

There has been much debate about the stability and function of S100A8 and S100A9 in the homodimer form. Some studies conducted on the two human proteins showed that S100A8 protein but not mRNA was absent after antisense S100A9 treatment (Kerkhoff *et al.*, 2005). In addition, S100A8 protein was absent in neutrophils of S100A9 null mice, although S100A8 mRNA was present (Hobbs *et al.*, 2003; Manitz *et al.*, 2003). The authors argued that the absence of S100A8 protein may be due to inefficient translation of the mRNA transcripts, or due to the instability of S100A8 protein in an environment that is deficient in S100A9 (Hobbs *et al.*, 2003). In a study by Rosenberger *et al.* (2007), human keratinocyte cells stably transfected with an expression construct for S100A8 showed up-regulation of S100A8 as well as endogenous S100A9 in these cells. Furthermore, down-regulation of S100A8 protein by siRNA correlated with a decrease in S100A9 protein levels and *vice versa* (Rosenberger *et al.*, 2007). The authors concluded

that their data "clearly demonstrate a coordinately regulated expression of the two calcium-binding proteins" (Rosenberger *et al.*, 2007). Supporting this idea are biophysical data which showed that human S100A8 and S100A9 preferentially form the heterodimeric complex, and unlike S100A9, S100A8 was unable to form stable, well-packed homodimers, which lead the authors to conclude that biological activity is restricted to S100A9 homodimers or the S100A8/S100A9 complex but not S100A8 homodimers (Hunter and Chazin, 1998).

The findings of the present study, however, suggest that the two murine proteins may not depend on each other for stability, since S100A9 but not S100A8 protein was present after establishing the expression of both genes into the COS-7 cells. The possibility of ubiquitin-dependent proteolysis of the S100A8 protein was also examined; however, it is unlikely that S100A8 is degraded by proteosomes in the stable cells since treatment with MG-132 did not reveal S100A8 protein. A better understanding of the dynamic processes involved in protein synthesis and degradation is necessary in order to explain the variation between S100A8 mRNA and protein levels observed in this study.

The results here are in agreement with the findings of Mou *et al.* (2006), who showed that S100A9 gene expression can be down-regulated in HL-60 cells by shRNA without demonstrating effects on S100A8. Others were unable to detect S100A9 protein following activation of murine RAW 264.7 and WEHI 265 macrophage cells with LPS, which induced S100A8 expression (Hu *et al.*, 1996; Xu *et al.*, 2001; Xu and Geczy, 2000). Stimulated murine primary fibroblasts have also been shown to express S100A8 in the absence of S100A9 (Rahimi *et al.*, 2005). Moreover, the homodimerization of murine

S100A8 and S100A9 has been confirmed by Propper *et al.* (1999), although the stability of the homodimers was reduced compared with the heterodimer.

In the original demonstration of the lethal gene deletion of murine S100A8, the expression of S100A8 was evident in trophobasts at 6.5 days post coitum (dpc), while S100A9 is not expressed until day 14 (Passey *et al.*, 1999a). In addition, only S100A9 is expressed in fetal liver beginning 10.5 dpc (Lagasse and Weisman, 1992; Lichanska *et al.*, 1999). Also, in contrast to S100A8, there was no up-regulation of S100A9 in UVA-irradiated murine fibroblasts in skin or in keratinocytes in culture (Grimbaldeston *et al.*, 2003). This mounting evidence suggests that co-expression of S100A8 and S100A9 is not a requirement for their stability in murine systems, reflecting possible functional differences between the human and mouse proteins. Recently, it was demonstrated that S100A8 is induced exclusively in murine macrophages with poly(I:C), a synthetic analog of dsRNA used to simulate viral infections (Endoh *et al.*, 2009). In contrast, S100A8 and S100A9 were co-induced by poly(I:C) in human monocytes (Endoh *et al.*, 2009), which confirms the differences in gene regulation of the two S100 proteins in mice and humans.

Several other studies have demonstrated that S100A8 and S100A9 are expressed independently in inflammatory conditions (Brun *et al.*, 1994; Hogg *et al.*, 1985, 1989; Odink *et al.*, 1987; Sorg *et al.*, 1992). The fact that trophoblasts in murine embryos express S100A8 in the absence of S100A9 and S100A8 null embryos do not survive (Passey *et al.*, 1999a), while S100A9 null mice appear normal (Hobbs *et al.*, 2003), is evidence for a non-redundant function for S100A8 protein in development. All of these findings suggest that murine S100A8 and S100A9 can be stable and have functions independent of the heterodimer complex.

4.5 KNOCKDOWN OF S100A8 WITH SHORT-HAIRPIN RNA

RNAi technology has become an effective tool for the knockdown of gene expression by mediating the degradation of the corresponding mRNA in a wide variety of target cells. The best approach to test shRNA efficiency *in vitro* is to measure constitutively-expressed genes in unstimulated cell systems following shRNA transfection (Endoh *et al.*, 2009). Primary neutrophils are known to constitutively express S100A8, but due to their short half-life in culture and low transfection ability (Yi *et al.*, 2004), alternative means for the validation of S100A8 suppression *in vitro* were sought. The proposed approach was to over-express S100A8 in COS-7 cells and subsequently suppress its expression using shRNAs based on the primary miR-30 miRNA, which have been shown to be more potent in silencing gene expression than traditional stem-loop shRNAs (Boden *et al.*, 2004; Silva *et al.*, 2005). Three different miR-30-based shRNAs targeting murine S100A8 were designed for this study and their efficacy tested. Sequence alignment confirmed that the hairpins are specific for murine S100A8.

4.5.1 Transient shRNA Transfections

For most applications of RNAi, it is sufficient if the silencing effect is only transient, long enough to be able to demonstrate the knockdown. However, this is largely dependent on both the efficiency of shRNA transfection and its expression in the nucleus (Luo and Saltzman, 2000). In this study, transient transfections of shRNAs presented with a very low efficiency, as revealed by fluorescence microscopy. Several factors are generally implicated in poor transfection efficiency, including the presence of media components such as serum and antibiotics (the transfection reagent may bring the antibiotics into the cells or the lipid-DNA complex may interact with the antibiotics

impacting its entrance into the cells). These factors could not have played a role in the low transfection efficiencies observed in this study since cells were transfected in fresh media deficient in antibiotics and serum. Geneticin (G418) selection, which was used to maintain S100A8 expression in the stable COS-7 cells, was also discontinued during the initial shRNA transfection period. Optimal transfection efficiency and subsequent cell viability also depend on a number of other experimental variables, including cell density, poor quality DNA and insufficient or excess amounts of DNA and/or transfection reagent. Experimental conditions were manipulated to account for these factors; however, attempts to improve the efficiency of the transient transfections were unsuccessful.

There are cellular and molecular barriers to transfection by cationic lipids. Studies have shown that cationic lipid-mediated gene transfer is an inefficient process (Zabner *et al.*, 1995). Interestingly, this is not due to the inability of the lipid-DNA complex to enter the cell, but rather the movement of DNA from the cytoplasm into the nucleus seems to be the major limitation to successful transfection (Escriou *et al.*, 2001; Zabner *et al.*, 1995). In a study by Carpentier *et al.* (2007), it was found that up to 48% of plasmid DNA delivered using polyethylenimine (PEI) was associated with the total cell extracts one day post-transfection, while only 1-2% was found in the nuclear fraction two days post-transfection. The authors suggested that the physiological state of the cell may limit nuclear translocation of plasmid DNA and/or its transcription (Carpentier *et al.*, 2007).

Upon entrance into the cells, the lipid-DNA complex may associate with endosomes, which represent yet another barrier to the efficiency of transfection (Zabner *et al.*, 1995). Following the entry of the complexes into the nucleus, the lipid and DNA must dissociate before the plasmid genes can be expressed. Godbey *et al.* (1999) reported

that the majority of plasmid DNA in the nucleus was still complexed with the transfection reagent. Accordingly, the transcriptional competency of plasmid DNA governs the expression of plasmid genes (Carpentier *et al.*, 2007). Thus, nuclear translocation and transcriptional barriers may have been major limitations to shRNA expression in the transient assays. It is worth noting that the use of viruses as vehicles for siRNA/shRNA expression has solved many of the barriers associated with lipid-mediated transfections (Horwitz, 1990), and should be considered for future studies.

4.5.2 S100A8 Expression Levels in Stable shRNA Cell Lines

One issue to consider with regards to the knockdown experiments is the shRNA transfection process itself, which may alter S100A8 expression because this gene appears to be induced in response to cell stress (Endoh *et al.*, 2009; Grimbaldeston *et al.*, 2003). Thus, cell lines stably-silencing the S100A8 gene are preferred. Because of this, as well as the difficulties encountered in transfecting the shRNAs transiently, cell lines stably-expressing the hairpins were generated for this study. The results show that one of the shRNA molecules, shRNA_mA8_65, was able to achieve a moderate level of suppression (62%) of S100A8 mRNA levels, confirming that shRNAs could effectively down-regulate S100A8 expression in an *in vitro* mammalian system. The other relevant hairpin, shRNA_mA8_250, did not reduce S100A8 mRNA levels sufficiently (25% knockdown), although the results were significant ($P<0.05$) compared to the control.

Visual analysis of GFP expression in stable shRNA cell lines revealed differences in the intensity of fluorescence in the cell population, which reflects differences in the numbers of plasmid copies that had been integrated and/or the efficiency of gene transcription, which may depend on the genomic site of integration of the shRNAs. To

confirm that the knockdown efficacy of each hairpin does not reflect differences in shRNA expression levels among treatment groups, GFP expression in each sample was measured quantitatively by real-time RT-PCR and correlated with shRNA expression levels. This approach is adopted by researchers since amplification of the shRNA sequence can be problematic due to the intrinsic secondary structure of the hairpin (Taxman *et al.*, 2006). In fact, a common difficulty encountered with shRNAs is that of confirming the sequence of the hairpin region (Taxman *et al.*, 2006), which has been shown to cause the elongation reactions to terminate prematurely due to the inability of the polymerase to read through the template (Guo *et al.*, 2005; Yu *et al.*, 2003). Dickins *et al.* (2005), who designed the original MSCV-LTRmiR30-PIG shRNA vector used here, assume the GFP-shRNA correlation in their study of synthetic microRNA precursors, in which they specify that: "GFP expression serves as a surrogate marker of shRNA production." The comparable expression levels of GFP between treatments confirmed that the level of knockdown achieved is representative of the efficacy and potency of each shRNA molecule used in this study.

Effective RNA silencing is capable of reducing target gene expression by 70% or greater, and can achieve close to complete suppression of the target gene (Ui-Tei *et al.*, 2004). However, RNAi in mammalian cells varies greatly between experiments, and depends to a large extent on the RNA duplexes used and their level of expression. As such, not all 19-23 bp duplexes will be able to cleave their target mRNA with the same efficacy, and consequently, bring about the same silencing effect. Broad differences in the efficacy of shRNA knockdown have been reported. For example, out of the 27 shRNA vectors that Taxman *et al.* (2006) evaluated, more than one-third achieved less

than 10% suppression, despite comparable high expression levels of GFP in these cells. The fact that they observed great variation in the efficacy of shRNAs designed against the same target genes lead to the conclusion that there is no simple biological answer for the differences in knockdown efficiency observed in their study (Taxman *et al.*, 2006).

There are a number of variables which may account for the low levels of suppression achieved by some shRNAs, including shRNA design, delivery system, stressed state of the cell and possibly cell type (Ui-Tei *et al.*, 2007). This is precisely why several candidate shRNAs must be screened to identify effective silencers. The variability in the efficacy of different shRNAs to knockdown the same target gene may be explained by the fact that different shRNAs exhibit different thermodynamic properties, such that the mature product of one hairpin may be better than another in interacting with its target mRNA. The formation of internal hairpins by the RNA molecules within RISC may also reduce the silencing ability of the shRNA (Khvorova *et al.*, 2003). S100A8 suppression in COS-7 cells over-expressing the gene may have also affected the levels of knockdown observed in this study. An imbalance between the up-regulation and suppression of S100A8 expression may have occured, much in the same way suggested earlier for simultaneous induction of S100A8 and inhibition by RNAi (Endoh *et al.*, 2009).

Poor shRNA design can be detrimental to the efficiency of the hairpin. For instance, a hairpin may target a region of the mRNA that is highly structured or bound by regulatory proteins, making the target sequence inaccessible, thereby reducing the effectiveness of the shRNA (Brown *et al.*, 2005; Overhoff *et al.*, 2005). Other factors may also dictate the efficacy of the shRNAs, including the plasmid copy number present, the genomic site of insertion (which can enhance or hinder expression of the hairpin, or

otherwise interfere with expression of nearby genes), and the possibility of degradation of the RNA duplexes. Furthermore, it is important to verify the shRNA sequences, since a mismatch of a single base pair within the target mRNA sequence can abolish the knockdown (Brummelkamp *et al.*, 2002; Miller *et al.*, 2003), though this has proven to be a difficult task due to the complex secondary structure of the hairpin (Taxman *et al.*, 2006). It would be advantageous to verify the sequences of the shRNAs used in this study to determine if the relatively low levels of suppression achieved reflect an inherent defect in the design of the shRNAs or due to other mechanisms.

The ability of shRNAs to target and degrade mRNA may also be limited by saturation of the nuclear export factor, exportin-5, which transports pre-miRNA and shRNA transcripts from the nucleus to the cytoplasm (Yi *et al.*, 2003). Over-expression of recombinant exportin-5 has been shown to restore shRNA function (Grimm *et al.*, 2006; Yi *et al.*, 2005). It is important to discuss further the study of Grimm *et al.* (2006). A large number of the shRNAs used in their study caused liver injury, and many of the mice died as a result (Grimm *et al.*, 2006). The authors suggested that the oversaturation of exportin-5 resulted in a global repression of miRNAs, including the miRNAs normally present in cells (Grimm *et al.*, 2006). Natural miRNAs regulate many important genes involved in cell differentiation, development and apoptosis (Winter *et al.*, 2009). It was concluded that shRNAs must be used in moderation to avoid the adverse (and sometimes lethal) consequences (Grimm *et al.*, 2006). Potential off-target effects and activation of the immune response are yet other reasons for avoiding a massive RNAi response (Manjunath *et al.*, 2009).

These conclusions may provide an explanation for the relatively low levels of silencing obtained with the miR-30-based shRNAs used in this study, in particular, shRNA-A8_250. In COS-7 cells in which a massive RNAi response was elicited, exportin-5 may have been saturated to the point that the naturally occurring miRNAs could no longer be exported out of the nucleus to carry out their normal functions. Consequently, this would result in cell death. Thus, the remaining cell population represents cells which produced only modest amounts of shRNA transcripts. This idea is in agreement with the report of Yi *et al.* (2005), who found that clones producing moderate levels of shRNA did not show oversaturation of exportin-5 or cell death. Other factors, such as components of the RISC complex itself, may also have an impact on the efficiency of RNAi. Two studies have reported saturation of RISC, in which RISC assembly was found to be inefficient and saturable by siRNAs *in vitro* (Bitko and Barik, 2001; Hutvagner *et al.*, 2004).

In conclusion, further optimization of shRNA design and a better understanding of RNAi strategies and the natural miRNA pathway are necessary to unravel the full potential of this technology.

5. CONCLUSIONS

S100A8 has been identified as a potential genetic modifier of the inflammatory processes in CF lung disease. Although the effects of S100A8 over-expression have been addressed previously (Newbigging *et al.*, manuscript submitted), the consequences of the suppression of S100A8 on the lung-specific inflammatory phenotype in CF have not been explored. The aim of this study was to establish an *in vitro* RNAi-mediated gene-knockdown model that could be utilized in future studies in mice.

Due to the difficulties encountered in showing S100A8 expression in P388D1 and 32D cells, and because concurrent induction and suppression was deemed unsuitable for RNAi experiments, these earlier cell systems were abandoned. It was decided that suppression of S100A8 would be best achieved in COS-7 cells stably transfected with the murine S100A8 gene. The study presented here showed the potential for using miRNA-based shRNAs to knockdown S100A8 expression *in vitro*. Maximal suppression of S100A8 was 62% with shRNA_mA8_65, whereas shRNA_mA8_250 achieved 25% suppression and the two control shRNAs had no effect, compared to the untransfected cells. Although S100A8 expression was successfully reduced, the levels of knockdown achieved were not impressive when considering the potential of this technology for silencing gene expression. Much effort has gone towards identifying criteria for effective shRNA design, and these require further consideration in future studies.

Studies regarding the coordinate expression of the two proteins as a requirement for their stability have been controversial. The findings in this report suggest that this interdependence may not be relevant to the mouse proteins, since S100A9 protein was detected in cells transfected exclusively with the murine S100A9 gene. The presence of

S100A8 mRNA transcripts in cells stable for both S100A8 and S100A9 expression without a corresponding increase in S100A8 protein levels suggests that the strong down-regulation of S100A8 protein may be due to post-transcriptional mechanisms that prevented the proper translation and/or folding of the S100A8 gene product. Difficulties in purifying the two proteins have no doubt contributed to the elusiveness of their functional properties since their original discovery nearly three decades ago.

Strategies for Future Consideration

Although statistically significant reduction in S100A8 mRNA levels was achieved with the two relevant shRNAs used in this study, these levels may not be obtainable (or be biologically or therapeutically significant) *in vivo*, owing to the complex physiology of living organisms and the difficulties in targeting and transfecting primary cells (Yi *et al.*, 2004). The use of retroviral and lentiviral delivery systems, which have been shown to be more efficient and effective at silencing gene expression than DNA-based shRNA vectors (Manjunath *et al.*, 2009), may provide better results for future *in vivo* studies. Lentiviral vectors offer higher transfection efficiencies (especially in difficult to transfect primary cells) and stable knockdown by integration into the host genome (Manjunath *et al.*, 2009). It may also be worthwhile to investigate the effectiveness of chemically-synthesized siRNAs to target S100A8 expression in mice. Chemical modifications of the siRNA duplexes prior to delivery have been shown to improve the stability of siRNAs *in vivo* while maintaining their silencing potency (Soutschek *et al.*, 2004).

Inadequate shRNA design may have contributed to the relatively low levels of knockdown achieved in this study. Since only two relevant shRNAs were tested, it would be advisable to test additional shRNA molecules targeting different regions of S100A8

mRNA. Combinations of shRNAs may also enhance the levels of knockdown that can be attained compared to treatment with individual shRNA molecules. Furthermore, direct assessment of shRNA expression (possibly by quantitative RT-PCR or Northern blotting) may also be advantageous to corroborate the efficacy of the shRNA molecules used in this study.

Assessing the knockdown efficacy of shRNAs in stable cell lines is time consuming, so ways of improving the efficiency of the shRNA transfections in transient assays will be valuable. This would especially be helpful if several other shRNAs targeting S100A8 were obtained and tested. Transfection efficiency is highly dependent on the cell type; thus, using a different cell line, such as HEK-293 or CHO cells, which are reportedly readily transfected with Lipofectamine™ 2000, may permit transient measurements of shRNA activity. The use of a more efficient transfection reagent and/or cell culture conditions may solve the nuclear translocation and transcriptional barriers.

Because S100A8 protein levels could not be analyzed in this study, the suppression of S100A8 expression still requires confirmation at the protein level. It was perplexing why S100A8 mRNA transcripts were present in the absence of S100A8 protein in cells stable for S100A8 and S100A8/S100A9 expression. Using a more sensitive technique for detecting S100A8 protein, such as an ELISA, may provide better results in the future. Moreover, it may be advantageous to employ an *in vitro* translation assay to determine if S100A8 protein could be efficiently produced by the pDream2.1 expression vector. One further recommendation is to over-express S100A8 in a different cell line, preferably a mouse cell line that is more likely to possess the appropriate machinery for S100A8 protein synthesis, folding and post-translational processing.

6. FUTURE DIRECTION

Functions of S100A8 have yet to be fully elucidated, especially since targeted disruption of the S100A8 gene is embryonic lethal (Passey *et al.,* 1999a). The ability to silence S100A8 will improve our understanding of its role and offer a better picture of the regulation of S100A8 as a potential therapeutic strategy for lung disease in CF. In this study, shRNAs were shown to induce sequence-specific silencing of murine S100A8 in mammalian cells, a finding which will be useful in future research of the protein function *in vitro* as well as in animal models.

Knockdown Studies in Mice

RNAi technology has recently become the method of choice for gene knockdown. The therapeutic potential of this technique may now be demonstrated *in vivo* by down-regulating S100A8 expression using shRNAs delivered in a DNA or viral vector. This would first be demonstrated in wild-type B6 mice followed by studies on B6-CF mice. Following established protocols, *in vivo* gene silencing can be achieved by treating mice with shRNAs beginning at 3-weeks of age (before the onset of the inflammatory lung phenotype) and until 2-months of age, at which time treatment effects on the lung pathology will be measured. Reduction of S100A8 (and consequently S100A9) levels, by 50% or greater, in the serum and lung tissue is expected to reduce the number of infiltrating neutrophils in the airways, and consequently, ameliorate the lung inflammatory phenotype in CF mice. These future studies open the possibilities for unraveling the mechanisms by which S100A8 influences the pathogenesis and progression of lung disease in CF.

Anticipated Challenges

Although shRNAs have been used successfully to induce silencing of gene expression in mice (Carmell *et al.*, 2003; Liu *et al.*, 1999; Tiscornia *et al.*, 2003), challenges to their *in vivo* applications still exist. Delivery of shRNA vectors may interrupt the natural RNAi machinery, trigger an interferon response (particularly in the case of viral-based vectors), lead to toxicity, or have off-target effects (Rao *et al.*, 2009). Appropriate dosages of shRNA and treatment duration and frequency must be established in way to induce efficient gene silencing and avoid deleterious consequences in the mice. In addition, once the shRNA molecule is delivered systemically, it must remain intact and avoid degradation in circulation, be able to specifically target and enter diseased cells and bring about gene silencing (Alexopoulou *et al.*, 2001). To target specific tissues, one possibility is to use a tissue-specific promoter to drive the expression of the shRNA (Rao *et al.*, 2009; Taxman *et al.*, 2006). Compared to siRNAs, shRNAs face the additional challenge of having to enter the nucleus, and be expressed and processed prior to incorporation into RISC. Furthermore, shRNA activity that is detrimental to the cell's integrity may elicit selective pressures against their stable expression. Thus, optimization of RNAi strategies (including specificity, stability, delivery, efficacy and toxicity) is crucial as this technology progresses towards clinical trials.

The fact that endogenous expression of vector-based shRNAs has been shown to induce silencing *in vitro* and *in vivo* affords the opportunity for the creation of transgenic animal models, and represents a potential therapeutic strategy for treating viral infections, cancer, inflammatory conditions and any disease processes involving changes in host gene expression.

7. REFERENCES

Aigner, A. (2006) Gene silencing through RNA interference (RNAi) *in vivo*: strategies based on the direct application of siRNAs. *J Biotech, 124*, 12-25.

Alexopoulou, L., Holt, A.C., Medzhitov, R., and Flavell, R.A. (2001) Recognition of double stranded RNA and activation of NF-kappa B by Toll-like receptor3. *Nature,* 413, 732-738.

Amarzguioui, M., and Prydz, H. (2004) An algorithm for selection of functional siRNA sequences. *Biochem Biophys Res Commun, 316*, 1050-1058.

Andersson, K.B., Sletten, K., Berntzen, M.B., Dale, I., Brandtzaeg, P., Jellum, E., and Fagerhol, M.K. (1988) The leukocyte L1 protein: identity with the cystic fibrosis antigen and the calcium-binding MRP-8 and MRP-14 macrophage components. *Scand J Immunol, 28*, 241-245.

Armstrong, D.S., Grimwood, K., Carlin, J.B., Carzino, R., Gutierrez, J.P., Hull, J., *et al.* (1997) Lower airway inflammation in infants and young children with cystic fibrosis. *Am J Respir Crit Care Med, 156*, 1197-204.

Balough, K., McCubbin, M., Weinberger, M., Smits, W., Ahrens, R., and Fick, R. (1995) The relationship between infection and inflammation in the early stages of lung disease from cystic fibrosis. *Pediatr Pulmonol, 20*, 63-70.

Barnes, P.J., and Karin, M. (1997) Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med,* 336, 1066-10671.

Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell, 116*, 281-297.

Barthe, C., Figarella, C., Carrere, J., and Guy-Crotte, O. (1991) Identification of "cystic fibrosis protein" as a complex of two calcium-binding proteins present in human cells of myeloid origin. *Biochim Biophys Acta, 1096*, 175-177.

Baudier, J., Glasser, N., and Gerard, D. (1986) Ions binding to S100 proteins. I. Calcium- and zinc-binding properties of bovine brain S100 alpha alpha, S100a (alpha beta), and S100b (beta beta) protein: Zn2+ regulates Ca2+ binding on S100b protein. *J Biol Chem, 261,* 8192-8203.

Bernstein, E., Denli, A., and Hannon, G.J. (2001) The rest is silence. *RNA, 7*, 1509-1521.

Berthier, S., Paclet, M.H., Lerouge, S., Roux, F., Vergnaud, S., Coleman, A.W., and Morel, F. (2003) Changing the conformation state of cytochrome b558 initiates NADPH oxidase activation: MRP8/MRP14 regulation. *J Biol Chem, 278,* 25499-25508.

Bhardwaj, R.S., Zotz, C., Zwadlo-Klarwasser, G., Roth, J., Goebeler, M., Mahnke, K., Falk, M., Meinardus-Hager, G., and Sorg C. (1992) The calcium-binding proteins MRP8 and MRP14 form a membrane-associated heterodimer in a subset of monocytes/macrophages present in acute but absent in chronic inflammatory lesions. *Eur J Immunol, 22,* 1891-1897.

Bitko, V., and Barik, S. (2001) Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. *BMC Microbiol, 1,* 34.

Bitko, V., Musiyenko, A., Shulyayeva, O., and Barik, S. (2005) Inhibition of respiratory viruses by nasally administered siRNA. *Nature Medicine, 11,* 50-55.

Blackwell, T.S., Stecenko, A.A., and Christman, J.W. (2001) Dysregulated NF-κB activation in cystic fibrosis: evidence for a primary inflammatory disorder. *Am J Physiol Lung Cell Mol Physiol, 281,* L69-70.

Bonfield, T.L., Konstan, M.W., and Berger, M. (1999) Altered respiratory epithelial cell cytokine production in cystic fibrosis. *J Allergy Clin Immunol, 104,* 72-78.

Bonfield, T.L., Panuska, J.R., Konstan, M.W., Hilliard, K.A., Hilliard, J.B., Ghnaim, H., and Berger, M. (1995) Inflammatory cytokines in cystic fibrosis lungs. *Am J Respir Crit Care Med, 152,* 2111-2118.

Brandtzaeg, P., Dale, I., and Fagerhol, M.K. (1987) Distribution of formalin-resistant myelomonocytic antigen (LI) in human tissues. II. Normal and aberrant occurence in various epithelia. *Am J Clin Pathol, 87,* 700-707.

Brodersen, D.E., Etzerodt, M., Madsen, P., Celis, J.E., Thogersen, H.C., Nyborg, J., and Kjeldgaard, M. (1998) EF-hands at atomic resolution: the structure of human psoriasin (S100A7) solved by MAD phasing. *Structure, 6,* 477- 489.

Brown, K.M., Chu, C.Y., and Rana, T.M. (2005) Target accessibility dictates the potency of human RISC. *Nat Struct Mol Biol, 12,* 469-470.

Brummelkamp, T.R., Bernards, R., and Agami, R. (2002a) A system for stable expression of short interfering RNAs in mammalian cells. *Science, 296,* 550-553.

Brummelkamp, T.R., Bernards, R., and Agami, R. (2002b) Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell 2,* 243-247.

Brun, J.G., Jonsson, R., and Haga, H.J. (1994) Measurement of plasma calprotectin as an indicator of arthritis and disease activity in patients with inflammatory rheumatic diseases. *J Rheumatol, 21,* 733-738.

Bumcrot, D., Manoharan, M., Koteliansky, V., and Sah, D.W. (2006) RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nat Chem Biol, 2,* 711-719.

Cai, X., Hagedorn, C.H., and Cullen, B.R. (2004) Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA, 10,* 1957-66.

Carmell, M.A., Zhang, L., Conklin, D.S., Hannon, G.J., and Rosenquist, T.A. (2003) Germline transmission of RNAi in mice. *Nat Struct Biol, 10,* 91-92.

Carpentier, E., Paris, S., Kamen, A.A., and Durocher, Y. (2007) Limiting factors governing protein expression following polyethylenimine-mediated gene transfer in HEK293-EBNA1 cells. *J Biotech, 128,* 268-280.

Chen, C.Z., Li, L., Lodish, H.F., and Bartel, D.P. (2004) MicroRNAs modulate hematopoietic lineage differentiation. *Science, 303,* 83-86.

Cheng, A.M., Byrom, M.W., Shelton, J., and Ford, L.P. (2005) Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res, 33,* 1290-1297.

Cho, R.J., Campbell, M.J., Winzeler, E.A., Steinmetz, L., Conway, A., Wodicka, L., Wolfsberg, T.G., Gabrielian, A.E., Landsman, D., Lockhart, D.J., *et al.* (1998) A genome-wide transcriptional analysis of the mitotic cell cycle. *Mol Cel, 2,* 65-73.

Chung, K.H., Hart, C.C., Al-Bassam, S., Avery, A., Taylor, J., Patel, P.D., Vojtek, A.B., and Turner, D.L. (2006) Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155. *Nucleic Acids Res, 34,* e53.

Collins, F.S. (1992) Cystic fibrosis: molecular biology and therapeutic implications. *Science, 256,* 774-779.

Collins, S.J., Ruscetti, F.W., Gallagher, R.E., and Gallo, R.C. (1979) Normal functional characteristics of cultured human promyelocytic leukemia cells (HL-60) after induction of differentiation by dimethylsulfoxide. *J Exp Med, 149,* 969-974.

Cornish, C.J., Devery, J.M., Poronnik, P., Lackmann, M., Cook, D.I., and Geczy, C.L. (1996) S100 protein CP-10 stimulates myeloid cell chemotaxis without activation. *J Cell Physiol, 166,* 427-437.

Corvol, H., Fitting, C., Chadelat, K., Jacquot, J., Tabary, O., Boule, M., *et al.* (2003) Distinct cytokine production by lung and blood neutrophils from children with cystic fibrosis. *Am J Physiol Lung Cell Mol Physiol 284,* L997-1003.

Dale, I., and Brandtzaeg, P. (1989) Expression of epithelial LI antigen as an immunohistochemical marker of squamous cell carcinomas of the lung. *Histopathology, 14,* 493-502.

Dale, I., Fagerhol, M.K., and Naesgaard, I. (1983) Purification and partial characterization of a highly immunogenic human leukocyte protein, the Li antigen. *Eur YBiochem, 134,* 1-6.

Davidson, D.J., and Rolfe, M. (2001) Mouse Models of Cystic Fibrosis. *Trends Genet, 17,* S29-37.

Dawe, C.J., and Potter, M. (1957) Morphologic and biologic progression of a lymphoid neoplasm of the mouse in vitro and in vivo. *Am J Pathol, 33,* 603.

Dean, R., Fu, S., Stocker, R., and Davies, M. (1997) Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J, 324,* 1-18

de Fougerolles, A.R., Manoharan, M., Meyers, R., and Vornlocher, H.P. (2005) RNA interference *in vivo*: toward synthetic small inhibitory RNA-based therapeutics. *Methods Enzymol, 392,* 278-296.

Delaney, S.J., Alton, E.W., Smith, S.N., Lunn, D.P., Farley, R., Lovelock, P.K., Thomson, S.A., Hume, D.A., Lamb, D., Porteous, D.J., *et al.* (1996) Cystic fibrosis mice carrying the missense mutation G551D replicate human genotype-phenotype correlations. *EMBO J, 15,* 955-963.

Devery, J.M., King, N.J. and Geczy, C.L. (1994) Acute inflammatory activity of the S100 protein CP-10. Activation of neutrophils *in vivo* and *in vitro. J Immunol, 152,* 1888-1897.

Dickins, R.A., Hemann, M.T., Zilfou, J.T., Simpson, D.R., Ibarra, I., Hannon, G.J., and Lowe, S.W. (2005) Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. *Nature Genetics 37,* 1289-1295.

Donato, R. (2003) Intracellular and extracellular roles of S100 proteins. *Microsc Res Tech, 60,* 540-551.

Donato, R. (1999) Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type. *Biochim Biophys Acta, 1450,* 191-231.

Donato, R. (1991) Perspectives in S100 protein biology. *Cell Calcium, 12,* 713-726.

Donato, R. (2001) S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. *Int J Biochem Cell Biol, 33,* 637-668.

Dorin, J.R., Novak, M., Hill, R.E., Brock, D.J.H., Secher, D.S., and Van Heyningen, D. (1987) A clue to the basic defect in cystic fibrosis from cloning the CF antigen gene. *Nature, 326,* 614-617.

Doussiere, J., Bouzidi, F. and Vignais, P.V. (2002) The S100A8/A9 protein as a partner for the cytosolic factors of NADPH oxidase activation in neutrophils. *Eur J Biochem, 269,* 3246-3255.

Downward, J. (2004) RNA interference. *BMJ, 328,* 1245-1248.

Drohat, A.C., Nenortas, E., Beckett, D., and Weber, D.J. (1997) Oligomerization state of S100B at nanomolar concentration determined by large-zone analytical gel filtration chromatography. *Protein Sci, 6,* 1577-1582.

Edgeworth, J., Gorman, M., Bennett, P., Freemont, P., and Hogg, N. (1991) Identification of p8, 14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells. *J Biol Chem, 266,* 7706-7713.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001a) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature, 411,* 494-498.

Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl. T. (2001b) Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. *Embo J, 20,* 6877-6888.

Emberley, E.D., Murphy, L.C., and Watson, P.H. (2004) S100 proteins and their influence on pro-survival pathways in cancer. *Biochem Cell Biol, 82,* 508-515.

Endoh, Y., Chung, Y.M., Clark, I.A., Geczy, C.L., Hsu, K. (2009) IL-10-Dependent S100A8 Gene Induction in Monocyte/Macrophages by Double-Starnded RNA. *J of Immunol, 182,* 2258-2268.

Engelhardt J.F., Zepeda M., Cohn J.A., Yankaskas J.R., and Wilson J.M. (1994) Expression of the cystic fibrosis gene in adult human lung. *J Clin Invest, 93,* 737-749.

Escriou, V., Carriere, M., Bussone, F.,Wils, P., and Scherman, D. (2001) Critical assessment of the nuclear import of plasmid during cationic lipid-mediated gene transfer. *J Gene Med 3,* 179-187.

Eue, I., Pietz, B., Storck, J., Klempt, M., and Sorg, C. (2000) Transendothelial migration of 27E10+ human monocytes. *Int Immunol, 12,* 1593-1604.

Fagerhol, M.K, Dale, I., and Anderson, I. (1980) Release and quantitation of a leucocyte derived protein (L1). *Scand J Haematol, 24,* 393.

Fanjul, M., Renaud, W., Merten, M., Guy-Crotte, O., Hollande, E., and Figarella, C. (1995) Presence of MRP8 and MRP14 in pancreatic cell lines: differential expression and localization in CFPAC-1 cells. *Am J Physiol, 268,* C1241-1251.

Fano, G., Marsili, V., Angelella, P., Aisa, M.C., Giambanco, I., and Donato, R. (1989) S-100a0 protein stimulates Ca2+-induced Ca2+ release from isolated sarcoplasmic reticulum vesicles. *FEBS Lett, 255,* 381-384.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature, 391,* 806-811.

Foell, D., Frosch, M., Sorg, C., Roth, J. (2004) Phagocyte-specific calcium-binding S100 proteins as clinical laboratory markers of inflammation. *Clin Chim Acta 344,* 37-51

Foell, D., and Roth, J. (2004) Proinflammatory S100 proteins in arthritis and autoimmune disease. *Arthritis Rheum, 50,* 3762-3771.

Foell, D., Wittkowski, H., Vogl, T., and Roth, J. (2007) S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. *J Leukoc Biol, 81,* 28-37.

Frosch, M., Strey, A., Vogl, T., Wulffraat, N.M., Kuis, W., Sunderkotter, C., Harms, E., Sorg, C., and Roth, J. (2000) Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. *Arthritis Rheum, 43,* 628-637.

Gebhardt, C., Breitenbach, U., Tuckermann, J.P., Dittrich, B.T., Richter, K.H., and Angel, P. (2002) Calgranulins S100A8 and S100A9 are negatively regulated by glucocorticoids in a c-Fosdependent manner and overexpressed throughout skin carcinogenesis. *Oncogene, 21,* 4266-4276.

Gebhardt, C., Nemeth, J., Angel, P., and Hess. J. (2006) S100A8 and S100A9 in inflammation and cancer. *Biochem Pharmacol, 72, 1622*-1631.

Geczy, C. (1996) Regultion and proinflammatory properties of the chemotactic protein CP-10. *Biochim Biophys Acta, 1313,* 246-252.

Gil, J. and Esteban, M. (2000) Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): Mechanism of action. Apoptosis, 5, 107-114.

Godbey, W.T., Barry, M.A., Saggau, P., Wu, K.K., and Mikos, A.G. (2000) Poly(ethylenimine)-mediated transfection: a new paradigm for gene delivery. *J Biomed Mater Res, 51,* 321-328.

Goebeler, M., Roth, J., Burwinkel, F., Vollmer, E., Bocker, W., and Sorg, C. (1994) Expression and complex formation of S100-like proteins MRP8 and MRP14 by macrophages during renal allograft rejection. *Transplantation, 58,* 355-361.

Goebeler, M., Roth, J., Henseleit, U., Sunderkötter, C., and Sorg C. (1993) Expression and complex assembly of calcium-binding proteins MRP8 and MRP14 during differentiation of murine myelomonocytic cells. *J Leukoc Biol, 53,* 11-18.

Greger, R., Mall, M., Leich, M., Ecke, D., Warth, R., Riedemann, N., and Kunzelmann, K. (1996) Regulation of epithelial ion channels by the cystic fibrosis transmembrane conductance regulator. *J Mol Med, 74,* 527-534.

Grimbaldeston, M.A., Geczy, C.L., Tedla, N., Finlay-Jones, J.J., and Hart, P.H. (2003) S100A8 induction in keratinocytes by ultraviolet A irradiation is dependent on reactive oxygen intermediates. *J Invest Dermatol, 121,* 1168-1174.

Grimm, D., Streetz, K.L., Jopling, C.L., Storm, T.A., Pandey, K. Davis, C.R., Marion, P., Salazar, F., and Kay, M.A. (2006) Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature, 441,* 537-541.

Guo, Y., Liu, J., Li, Y.H., Song, T.B., Wu, J., Zheng, C.X., Xue, C.F. (2005) Effect of vector-expressed shRNAs on hTERT expression. *World J Gastroenterol, 11,* 2912-2915.

Gurzov, E.N., and Izquierdo, M. (2006) RNA interference against Hec1 inhibits tumor growth in vivo. *Gene Ther, 13,* 1-7.

Hamilton, A, and Baulcombe, D. (1999). "A species of small antisense RNA in post-transcriptional gene silencing in plants." *Science, 286,* 950-952.

Hammerle A.F., Tatschl C., Jatzko S., *et al.* (1997) The lung as an inflammatory organ. *Acta Anaesthesiol Scand Suppl, 111,* 59-64.

Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature, 404,* 293-296.

Hammond, S.M., Caudy, A.A., and Hannon, G.J. (2001) Post-transcriptional gene silencing by double-stranded RNA. *Nat Rev Genet, 2,* 110-119.

Hannon, G.J., and Rossi, J.J. (2004). Unlocking the potential of the human genome with RNA interference. *Nature, 431,* 371-378.

Harborth, J., Elbashir, S.M., Bechert, K., Tuschl, T., and Weber, K. (2001) Identification of essential genes in cultured mammalian cells using small interfering RNAs. *J Cell Sci, 114,* 4557-4565.

Harrison, C.A., Raftery, M.J., Walsh, J., Alewood, P., Iismaa, S.E., Thliveris, S., and Geczy, C.L. (1999) Oxidation regulates the inflammatory properties of the murine S100 protein S100A8. *J Biol Chem, 274,* 8561-8569.

Haslett, C. (1999) Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *Am J Respir Crit Care Med, 160*, S5-11.

Haston, C.K., McKerlie, C., Newbigging, S., Corey, M., Rozmahel, R. and Tsui, L.C. (2002) Detection of Modifier Loci Influencing the Lung Phenotype of Cystic Fibrosis Knockout Mice. *Mamm Genome, 13,* 605-613.

Heidel, J.D., Hu, S., Liu, X.F., Triche, T.J., and Davis, M.E. (2004) Lack of interferon response in animals to naked siRNAs. *Nat Biotechnol, 22,* 1579-1582.

Heizmann, C.W. (1992) Calcium-binding proteins: basic concepts and clinical implications. *Gen Physiol Biophys, 11,* 411-425.

Heizmann, C.W. (2002a) The multifunctional S100 protein family. *Methods Mol Biol, 172,* 69-80.

Heizmann, C.W., and Cox, J.A. (1998) New perspectives on S100 proteins: a multi-functional Ca(2+)-, Zn(2+)- and Cu(2+)-binding protein family. *Biometals, 11,* 383-397.

Heizmann, C.W., Fritz, G., and Schafer, B.W. (2002b) S100 proteins: structure, functions and pathology. *Front Biosci, 7,* 1356-1368.

Henson, P.M. (2003) Possible roles for apoptosis and apoptotic cell recognition in inflammation and fibrosis. *Am J Respir Cell Mol Biol, 29*, S70-76.

Hessian, P.A., Edgeworth, J., and Hogg, N. (1993) MRP-8 and MRP-14, two abundant Ca(2+)-binding proteins of neutrophils and monocytes. *J Leukoc Biol, 53*, 197-204.

Hetland, G., Talgo, G.J., and Fagerhol, M.K. (1998) Chemotaxins C5a and fMLP induce release of calprotectin (leucocyte L1 protein) from polymorphonuclear cells in vitro. *Mol Pathol, 51,* 143-148.

Hermani, A., De Servi, B., Medunjanin, S., Tessier, P.A., and Mayer, D. (2006) S100A8 and S100A9 activate MAP kinase and NF-kappaB signaling pathways and trigger translocation of RAGE in human prostate cancer cells. *Exp Cell Res, 312,* 184-197.

Hobbs, J.A., May, R., Tanousis, K., McNeill, E., Mathies, M., and Gebhardt, C., Henderson R, Robinson, M.J., and Hogg, N. (2003) Myeloid cell function in MRP-14 (S100A9) null mice. *Mol Cell Biol, 23,* 2564-2576.

Hogg, N., Allen, C., and Edgeworth, J. (1989) Monoclonal antibody 5.5 reacts with p8,14, a myeloid molecule associated with some vascular endothelium. *Eur J Immunol, 19,* 1053-1061.

Hogg, N., Palmer, D.G., and Revell, P.A. (1985) Mononuclear phagocytes of normal and rheumatoid synovial membrane identified by monoclonal antibodies. *Immunology, 56,* 673-681.

Horwitz, M.S. (1990) Adenoviridae and their replication. In: Fields, B.N. and Knipe, D.M., Editors, 1990. *Fields Virology* (2nd ed.), NY, NY: Raven Press, pp. 1679-1721.

Hsieh, A., Bo, R., Manola, J., Vazquez, F., Bare, O., Khvorova, A., Scaringe, S., and Sellers, W. (2004) A library of siRNA duplexes targeting the phosphoinositide 3-kinase pathway: determinants of gene silencing for use in cell-based screens. *Nucleic Acids Res, 32,* 893-901.

Hsu, K., Passey, R.J., Endoh, Y., Rahimi, F., Youssef, P., Yen, T., and Geczy, C.L. (2005) Regulation of S100A8 by Glucocorticoids. *J Immunol, 174,* 2318-2326.

Hu, S., Harrison, C., Xu, K., Cornish, C., and Geczy, C. (1996) Induction of the chemotactic S100 protein, CP-10, in monocyte/macrophages by lipopolysaccharide. *Blood, 87,* 3919-3928.

Hunter, M.J., and Chazin, W.J. (1998) High Level Expression and Dimer Characterization of the S100 EF-hand Proteins, Migration Inhibitory Factor-related Proteins 8 and 14. *J Biol Chem, 273,* 12427-12435.

Huppi, K., Martin, S.E., Caplen, N.J. (2005) Defining and assaying RNAi in mammalian cells. *Mol Cell, 17,* 1-10.

Hutvagner, G., Simard, M.J., Mello, C.C., and Zamore, P.D. (2004) Sequence-specific inhibition of small RNA function. *PLoS Biol, 2,* E98.

Ishiguro, H., Steward, M.C., Naruse, S., Ko, S.B., Goto, H., Case, R.M., Kondo, T., Yamamoto, A. (2009) CFTR functions as a bicarbonate channel in pancreatic duct cells. *J Gen Physiol, 133,* 315-326.

Ishikawa, K., Nakagawa, A., Tanaka, I., Suzuki, M., and Nishihira, J. (2000) The structure of human MRP8, a member of the S100 calcium-binding protein family, by MAD phasing at 1.9 A resolution. *Acta Crystallogr D Biol Crystallogr, 56,* 559-566.

Itou, H., Yao, M., Fujita, I., Watanabe, N., Suzuki, M., Nishihira, J., and Tanaka, I. (2002) The crystal structure of human MRP14 (S100A9), a Ca2+-dependent regulator protein in inflammatory process. *J Mol Biol, 316,* 265-276.

Ivanova, N., Dobri, R., Lu, R., Kotenko, I., Levorse, J., DeCoste, C., Schafer, X., Lun, Y., and Lemischka, I.R. (2006) Dissecting self-renewal in stem cells with RNA interference. *Nature, 442,* 533-538.

Jacobs, A.T., and Ignarro, L.J. (2003) Cell density-enhanced expression of inducible nitric oxide synthase in murine macrophages mediated by interferon-beta. *Nitric Oxide, 8,* 222-230.

Jensen, F.C., Girardi, A.J., Gilden, R.V., and Koprowski, H. (1964) Infection of human and simian tissue cultures with rous sarcoma virus. *Proc Natl Acad Sci USA 52,* 53-59.

Jiang, G., Li, J., Zeng, Z., and Xian, L. (2006) Lentivirus-mediated gene therapy by suppressing survivin in BALB/c nude mice bearing oral squamous cell carcinoma. *Cancer Biol Ther, 5,* 435-440.

Kammouni, W., Figarella, C., Marchand, S., and Merten, M. (1997) Altered cytokine production by cystic fibrosis tracheal gland serous cells. *Infect Immun, 65,* 5176-5183.

Kawasaki, H., Nakayama, S., and Kretsinger, R.H. (1998) Classification and evolution of EF-hand proteins. *Biometals, 11,* 277-295.

Kennerdell, J.R. and Carthew, R.W. (2000) Heritable gene silencing in *Drosophila* using double-stranded RNA. *Nat Biotechnol, 18,* 896-898.

Kent, G., Iles, R., Bear, C.E., Huan, L.J., Griesenbach, U., McKerlie, C., Frndova, H., Ackerley, C., Gosselin, D., Radzioch, D., O'Brodovich, H., Tsui, L.C., Buchwald, M., and Tanswell, A.K. (1997) Lung disease in mice with cystic fibrosis. *J Clin Invest, 100,* 3060-3069.

Kerem, B.S., Rommens, J.M., Buchanan, J.A., Markiewicz, D., Cox, T.K., Chakravart, A., Buchwald, M., and Tsui, L.C. (1989) Identification of the cystic fibrosis gene: genetic analysis. *Science, 245,* 1073-1080

Kerkhoff, C., Klempt, M., and Sorg, C. (1998) Novel insights into structure and function of S100A8 and S100A9. *Biochim Biophys Acta 1448,* 200-211.

Kerkhoff, C., Klempt, M., Kaever, V., and Sorg, C. (1999) The two calcium-binding proteins, S100A8 and S100A9, are involved in the metabolism of arachidonic acid in human neutrophils. *J Biol Chem, 274,* 32672-32679.

Kerkhoff, C., Nacken, W., Benedyk, M., Dagher, M.C., Sopalla, C., and Doussiere, J. (2005) The arachidonic acid-binding protein S100A8/A9 promotes NADPH oxidase activation by interaction with $p67^{phox}$ and Rac-2. *FASEB J, 19,* 467-469.

Kerkhoff, C., Sorg, C., Tandon, N.N., and Nacken, W. (2001) Interaction of S100A8/S100A9-arachidonic acid complexes with the scavenger receptor CD36 may facilitate fatty acid uptake by endothelial cells. *Biochemistry, 40,* 241-248.

Khvorova, A., Reynolds, A., Jayasena, S.D. (2003) Functional siRNAs and miRNAs exhibit strand bias. *Cell, 115,* 209-216.

Kido, J., Hayashi, N., Kataoka, M., and Nagata, T. (2005) Calprotectin expression in human monocytes: induction by porphyromonas gingivalis lipopolysaccharide, tumor necrosis factor-alpha, and interleukin-1beta. *J Periodontol, 76,* 437-442.

Kim, D.H. and Rossi, J.J. (2007) Strategies for silencing human disease using RNA interference. *Nature Rev Genet, 8,* 173-184.

Klempt, M., Melkonyan, H., Nacken, W., Wiesmann, D., Holtkemper, U., and Sorg, C. (1997) The heterodimer of the Ca2+-binding proteins MRP8 and MRP14 binds to arachidonic acid. *FEBS Lett, 408,* 81-84.

Kligman, D., and Hilt, D.C. (1988) The S100 protein family. *Trends Biochem Sci, 13,* 437-443.

Knowles, M.R., Stutts, M.J., Spock, A., *et al.* (1983) Abnormal ion permeation through cystic fibrosis respiratory epithelium. *Science, 221,* 1067-1070.

Konstan, M.W., and Berger, M. (1997) Current understanding of the inflammatory process in cystic fibrosis: onset and etiology. *Pediatr Pulmonol, 24,* 137-142.

Koren, H.S., Handwerger, B.S., and Wunderlich, J.R. (1975) Identification of macrophage-like characteristics in cultured murine tumor line. *J Immunol, 114,* 894-897.

Korndörfer, I.P., Brueckner, F., and Skerra, A. (2007) The crystal structure of the human (S100A8/S100A9)2 heterotetramer, calprotectin, illustrates how conformational changes of interacting alpha-helices can determine specific association of two EF-hand proteins. *J Mol Biol, 370,* 887-898.

Kretsinger, R.H. (1987) Calcium coordination and the calmodulin fold: divergent versus convergent evolution. *Cold Spring Harb Symp Quant Biol, 52,* 499-510.

Kube, E., Becker, T., Weber, K., and Gerke. V. (1992) Protein-protein interaction studied by sitedirected mutagenesis. Characterization of the annexin II-binding site on p11, a member of the S100 protein family. *J Biol Chem, 267,* 14175.

Kumar, R.K., Yang, Z., Bilson, S., Thliveris, S., Cooke, B.E. and Geczy, C.L. (2001) Dimeric S100A8 in human neutrophils is diminished after phagocytosis. *J Leukoc Biol, 70,* 59-64.

Kunath, T., Gish, G., Lickert, H., Jones, N., Pawson, T., and Rossant, J. (2003) Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. *Nat Biotechnol, 21,* 559-561.

Kunz, M., Roth, J., Sorg, C., and Kolde, G. (1992) Epidermal expression of the calcium binding surface antigen 27E10 in inflammatory skin diseases. *Arch Dermatol Res, 284,* 386-390.

Kunzelmann, K. (2001) CFTR: interacting with everything? *News Physiol Sci,* 16, 167-170.

Kuwayama, A., Kuruto, R., Horie, N., Takeishi, K., and Nozawa, R. (1993) Appearance of nuclear factors that interact with genes for myeloid calcium binding proteins (MRP-8 and MRP-14) in differentiated HL-60 cells. *Blood, 81,* 3116-3121.

Lackmann, M., Cornish, C. J., Simpson, R. J., Moritz, R. L., Geczy, C. L. 1992. Purification and structural analysis of a murine chemotactic cytokine (CP-10) with sequence homology to S100 proteins. *J Biol Chem, 267,* 7499-7504.

Lackmann, M., Rajasekariah, P., Iismaa, S.E., Jones, G., Cornish, C.J., Hu, S., Simpson, R.J., Moritz, R.L., and Geczy, C.L. (1993) Identification of a chemotactic domain of the pro-inflammatory S100 protein CP-10. *J Immunol, 150,* 2981-2991.

Lagasse, E., and Clerc, R.G. (1988) Cloning and expression of two human genes encoding calcium-binding proteins that are regulated during myeloid differentiation. *Mol Cell Biol, 8,* 2402-2410.

Lagasse, E., and Weissman, I.L. (1992) Mouse MRP8 and MRP14, two intracellular calciumbinding proteins associated with the development of the myeloid lineage. *Blood, 79,* 1907-1915.

Lee, N.S., Dohjima, T., Bauer, G., Li, H., Li, M.J., Ehsani, A., Salvaterra, P., and Rossi, J. (2002) Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nature Biotech, 20,* 500-505.

Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell, 75,* 843-854.

Lee, Y.S., Nakahara, K., Pham, J.W., Kim, K., He, Z., Sontheimer, E.J., and Carthew, R.W. (2004) Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell, 117,* 69-81.

Leenen, P.J., Jansen, A.M., and van Ewijk, W. (1986) Murine macrophage cell lines can be ordered in a linear differentiation sequence. *Differentiation, 32,* 157-164.

Lemarchand, P., Vaglio, M., Mauel, J., and Markert, M. (1992) Translocation of a small cytosolic calcium-binding protein (MRP-8) to plasma membrane correlates with human neutrophil activation. *J Biol Chem, 267,* 19379-19382.

Leukert, N., Vogl, T., Strupat, K., Reichelt, R., Sorg, C., and Roth, J. (2006) Calcium-dependent tetramer formation of S100A8 and S100A9 is essential for biological activity. *J Mol Biol, 359,* 961-972.

Lewis, J., Melrose, H., Bumcrot, D., Hope, A., Zehr, C., Lincoln, S., Braithwaite, A., He, Z., Ogholikhan, S., Hinkle, K., Kent, C., *et al.* (2008) In vivo silencing of alpha-synuclein using naked siRNA. *Mol Neurodegene, 3,* 19.

Ley, K. (1996) Molecular mechanisms of leukocyte recruitment in the inflammatory process. *Cardiovasc Res, 32,* 733-742.

Li, B., Tang, Q., Cheng, D., Qin, C., Xie, F., Wei, Q., Xu, J., Liu, Y., Zheng, B., Woodle, M., Zhong, N., and Lu, P. (2005) Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. *Nature Medicine, 9,* 944-951.

Lichanska, A.M., Browne, C.M., Henkel, G.W., Murphy, K.M., Ostrowski, M.C., McKercher, S.R., Maki, R.A. and Hume, D.A. (1999) Differentiation of the mononuclear phagocyte system during mouse embryogenesis: the role of transcription factor PU.1. *Blood, 94,* 127-138.

Linsdell, P., Tabcharani, J.A., Rommens, J.M., Hou, Y.X., Chang, X.B., Tsui, L.C., Riordan, J.R., and Hanrahan, J.W. (1997) Permeability of wild-type and mutant cystic fibrosis transmembrane conductance regulator chloride channels to polyatomic anions. *J Gen Physiol, 110,* 355-364.

Liu, F., Song, Y., and Lui. D. (1999) Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther, 6,* 1258-1266.

Lugering, N., Kucharzik, T., Lugering, A., Winde, G., Sorg, C., Domschke, W., and Stoll, R. (1997) Importance of combined treatment with IL-10 and IL-4, but not IL-13, for inhibition of monocyte release of the Ca(2+)-binding protein MRP8/14. *Immunology, 91,* 130-134.

Lundgren, J.D., and Vestbo, J. (1995) The pathophysiological role of mucus production in inflammatory airway diseases. *Respir Med, 89,* 315-316.

Luo, D., and Saltzman, W.M., (2000) Synthetic DNA delivery systems. *Nat Biotechnol, 18,* 33-37.

Manitz, M., Horst, B., Seeliger, S., Strey, A., Skryabin, B., Gunzer, M., Frings, W., Schönlau, F., Roth, J., Sorg, C., and Nacken, W. (2003) Loss of S100A9 (MRP14) results in reduced interleukin-8-induced CD11b surface expression, a polarized microfilament system, and diminished responsiveness to chemoattractants in vitro. *Mol Cell Biol, 23,* 1034-1043.

Manjunath, N., Wu, H., Subramanya, S., and Shankar, P. (2009) Lentiviral delivery of short hairpin RNAs. *Advanced Drug Delivery Reviews, 61,* 732-745.

Marenholz, I., Heizmann, C.W., and Fritz, G. (2004) S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). *Biochem Biophys Res Commun, 322,* 1111-1122.

Marissen, W.E., and Lloyd, R.E. (1998) Eukaryotic translation initiation factor 4G is targeted for proteolytic cleavage by caspase3 during inhibition of translation in apoptotic cells. *Mol Cell Biol, 18,* 7565-7574.

Matranga, C., Tomari, Y., Shin, C., Bartel, D.P., and Zamore, P.D. (2005) Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell, 123,* 607-620.

Matzke, M.A., and Birchler, J.A. (2005) RNAi-mediated pathways in the nucleus. *Nat Rev Genet, 6,* 24-35.

McCaffrey, A,P., Meuse, L., Pham, T.T., Conklin, D.S., Hannon, G.J., Kay, M.A. (2002) RNA interference in adult mice. *Nature, 418,* 38-39.

McCormick, M., Rahimi, M.F., Bobryshev, Y.V., Gaus, K., Zreiqat, H., Cai, H., Lord, R.S., and Geczy, C.L. (2005) S100A8 and S100A9 in human arterial wall. Implications for atherogenesis. *J Biol Chem, 280,* 41521-41529.

McElvaney, N.G., and Crystal, R.G. (1999) Proteases and lung injury. The lung. New York, NY: Raven, pp. 2205-2218.

McKeon, D.J., Condliffe, A.M., Cowburn, A.S., Cadwallader, K.C., Farahi, N., Bilton, D., and Chilvers, E.R. (2008) Prolonged survival of neutrophils from patients with Delta F508 CFTR mutations. *Thorax,* 63, 660-661.

Miller, V.M., Xia, H., Marrs, G.L., Gouvion, C.M., Lee, G., Davidson, and B.L., Paulson, H.L.(2003) Allele-specific silencing of dominant disease genes. *Proc Natl Acad Sci USA, 100,* 7195-7200.

Miyagishi, M., and Taira, K. (2002) U6 promoter-driven siRNAs with four uridine 3' overhangs effectively suppress targeted gene expression in mammalian cells. *Nature Biotechnology, 20,* 497-500.

Moore, B.W. (1965) A soluble protein characteristic of the nervous system. *Biochem Biophys Res Commun, 19,* 739-744.

Mork, G., Schjerven, H., Mangschau, L., Soyland, E., and Brandtzaeg, P. (2003) Proinflammatory cytokines upregulate expression of calprotectin (L1 protein, MRP-8/MRP-14) in cultured human keratinocytes. *Br J Dermatol, 149,* 484-491.

Most, P., Boerries, M., Eicher, C., Schweda, C., Ehlermann, P., Pleger, S.T., *et al.* (2003) Extracellular S100A1 protein inhibits apoptosis in ventricular cardiomyocytes via activation of the extracellular signal-regulated protein kinase 1/2 (ERK1/2). *J Biol Chem, 278,* 48404-48412.

Mou, Z., Zhou, J., Li, J., Zou, L., Zhou, W., and Wu, Y. (2006) Expression of small hairpin RNAs for S100A9 used in the protein function research. *Leukemia Research, 30,* 1013-1017.

Muhlebach, M.S., Stewart, P.W., Leigh, M.W., and Noah, T.L. (1999) Quantitation of inflammatory responses to bacteria in young cystic fibrosis and control patients. *Am J Repir Crit Care Med, 160,* 186-191.

Murao, S., Collart, F.R., and Huberman, E. (1989) A protein containing the cystic fibrosis antigen is an inhibitor of protein kinases. *J Biol Chem, 264,* 8356-8360.

Murao, S., Collart, F., and Huberman, E. (1990) A protein complex expressed during terminal differentiation of monomyelocytic cells is an inhibitor of cell growth. *Cell Growth Differ, 1,* 447-454.

Murao, S., Gemmell, M., Callaham, M., Anderson, N., and Huberman, E. (1983) Control of macrophage differentiation in human promyelocytic HL-60 leukemia cells by 1,25-dihydroxyvitamin D3 and phorbol-12-myristate-13-acetate. *Cancer Res, 43,* 4989-4996.

Nacken, W., and Kerkhoff. C., (2007) The hetero-oligomeric complex of the S100A8/S100A9 protein is extremely protease resistant. *FEBS Lett, 581,* 5127-5130.

Nacken, W., Roth, J., Sorg, C., and Kerkhoff, C. (2003) S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. *Microsc Res Tech, 60,* 569-580.

Nacken, W., Sopalla, C., Propper, C., Sorg, C., and Kerkhoff, C. (2000) Biochemical characterization of the murine S100A9 (MRP14) protein suggests that it is functionally equivalent to its human counterpart despite its low degree of sequence homology. *Eur J Biochem, 267,* 560-565.

Napoli C., Lemieux C., and Jorgensen R. (1990) "Introduction of a chalcone synthase gene into Petunia results in reversible co-suppression of homologous genes *in trans*". *Plant Cell, 2,* 279-289.

Newbigging, S., *et al.* (manuscript submitted). Phenotypic Investigation of mS100A8 over-expression in murine lung.

Newton, R.A., and Hogg, N. (1998) The human S100 protein MRP-14 is a novel activator of the beta 2 integrin Mac-1 on neutrophils. *J Immunol, 160,* 1427-1435.

Odink, K., Cerletti, N., Bruggen, J., Clerc, R. G., Tarcsay, L., Zwadlo, G., Gerhards, G., Schlegel, R., and Sorg, C. (1987) Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. *Nature, 330,* 80-82.

Oppenheimer, E.H., and Esterly J.R. (1975) Pathology of cystic fibrosis review of the literature and comparison with 146 autopsied cases. *Perspect Pediatr Pathol,* 2, 241-278.

Overhoff, M., Alken, M., Far, R.K., Lemaitre, M., Lebleu, B., Sczakiel, G., and Robbins, I. (2005) Local RNA target structure influences siRNA efficacy: a systematic global analysis. *J Mol Biol, 348,* 871-881.

Paddison, P., Caudy, A.A., and Hannon, G.J. (2002b) Stable suppression of gene expression in mammalian cells by RNAi. *Proc Natl Acad Sci, 99,* 1443-1448.

Pain, V.M. (1996) Initiation of protein synthesis in eukaryotic cells. *Eur J Biochem, 236,* 747-771.

Passey, R.J., Williams, E., Lichanska, A.M., Wells, C., Hu, S., Geczy, C.L., Little, M.H., and Hume, D.A. (1999a) A null mutation in the inflammation-associated S100 protein S100A8 causes early resorption of the mouse embryo. *J Immunol, 163,* 2209-2216.

Passey, R.J., Xu, K., Hume, D., and Geczy, C. (1999b) S100A8: emerging functions and regulation. *J Leuk Biol, 66,* 549-556.

Paul, C.P., Good, P.D., Winer, I., and Engelke, D.R. (2002) Effective expression of small interfering RNA in human cells. *Nat Biotechnol, 20,* 505-508.

Pfeffer, K.D., Huecksteadt, T.P., and Hoidal, J.R. (1993) Expression and regulation of tumor necrosis factor in macrophages from cystic fibrosis patients. *Am J Respir Cell Mol Biol, 9,* 511-519.

Pilewski, J.M., and Frizzell, R.A. (1999) Role of CFTR in Airway Disease. *Physiol Rev, 79,* S215-255.

Propper, C., Huang, X., Roth, J., Sorg, C., and Nacken, W. (1999) Analysis of the MRP8-MRP14 protein-protein interaction by the two-hybrid system suggests a prominent role of the C-terminal domain of S100 proteins in dimer formation. *J Biol Chem, 274,* 183-188.

Pushparaj, P.N., Aarthi, J.J., Manikandan, and Kumar, S.D. (2008) siRNA, miRNA, and shRNA: *in vivo* Applications. *J Den Res, 87,* 992-1003.

Quinton, P.M. (1983) Chloride impermeability in cystic fibrosis. *Nature, 301,* 421- 422.

Raftery, M.J., and Geczy, C.L. (1998) Identification of noncovalent dimeric complexes of the recombinant murine S100 protein CP10 by electrospray ionization mass spectrometry and chemical cross-linking. *J Am Soc Mass Spectrom, 9,* 533-539.

Rahimi, F., Hsu, K., Endoh, Y., and Geczy, C.L. (2005) FGF-2, IL-1beta and TGF-beta regulate fibroblast expression of S100A8. *Febs J, 272,* 2811-2827.

Rammes, A., Roth, J., Goebeler, M., Klempt, M., Hartmann, M., and Sorg, C. (1997) Myeloid-related protein (MRP) 8 and MRP14, calcium-binding proteins of the S100 family, are secreted by activated monocytes via a novel, tubulin-dependent pathway. *J Biol Chem, 272,* 9496-9502.

Rana, T.M. (2007) Illuminating the silence: understanding the structure and function of small RNAs. *Nat Rev Mol Cell Biol, 8,* 23-36.

Rao, D.D., Vorhies, J.S., Senzer, N., and Nemunaitis, J. (2009) siRNA vs. shRNA: Similarities and differences. *Advanced Drug Delivery Reviews, 61,* 746-759.

Ratcliff, R., Evans, M.J., Cuthbert, A.W., MacVinish, L.J., Foster, D., Anderson, J.R., and Colledge, W.H. (1993) Production of a severe cystic fibrosis mutation in mice by gene targeting. *Nat Genet, 4,* 35-41.

Ratjen, F., and Doring, G. (2003) Cystic fibrosis. *Lancet, 361,* 681-689.

Ravasi, T., Hsu, K., Goyette, J., Schroder, K., Yang, Z., Rahimi, F., Miranda, L.P., Alewood, P.F., Hume, D.A., and Geczy, C. (2004) Probing the S100 protein family through genomic and functional analysis. *Genomics, 84,* 10-22.

Renaud, W., Merten, M., and Figarella, C. (1994) Increased coexpression of CFTR and S100 calcium binding proteins MRP8 and MRP14 mRNAs in cystic fibrosis human tracheal gland cells. *Biochem Biophys Res Commun, 201,* 1518-1525.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W.S., and Khvorova, A. (2004) Rational siRNA design for RNA interference. *Nat Biotechnol, 22,* 326-330.

Ridinger, K., Ilg, E.C., Niggli, F.K., Heizmann, C.W., and Schafer, B. (1998) Clustered organization of S100 genes in human and mouse. *Biochim Biophys Acta, 1448,* 254-263.

Riordan, J.R. (1993) The Cystic Fibrosis Transmembrane Conductance Regulator. *Annu Rev Physiol, 55,* 609-630.

Riordan, J., Rommens, J., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J., Drumm, M., Iannuzzi, M., Collins, F., and Tsui, L. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science,* 245, 1066-1073.

Rommens, J.M., Iannuzzi, M.C., Kerem, B., Drumm, M.L., Melmer, G., Dean, M., Rozmahel, R., Cole, J.L., Kennedy, D., Hidaka, N., Zsiga, M., Buchwald, M., Riordan, J.R., Tsui, L.C., and Collins, F.S. (1989) Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science, 245,* 1059-1065.

Rosenberger, S., Thorey, I.S., Werner, S., and Boukamp, P. (2007) A novel regulator of telomerase. S100A8 mediates differentiation-dependent and calcium-induced inhibition of telomerase activity in the human epidermal keratinocyte line HaCaT. *J Biol Chem, 282,* 6126-6135.

Rosenfeld, M., Gibson, R., McNamara, S., Emerson, J., Burns, J., Castile, R., Hiatt, P., McCoy, K., Wilson, C., Inglis, A., *et al.* (2001) Early pulmonary infection, inflammation, and clinical outcomes in infants with cystic fibrosis. *Pediatr Pulmonol, 32,* 356-366.

Roth, J., Burwinkel, F., van den Bos, C., Goebeler, M., Vollmer, E., and Sorg, C. (1993a) MRP8 and MRP14, S-100-like proteins associated with myeloid differentiation, are translocated to plasma membrane and intermediate filaments in a calcium-dependent manner. *Blood, 82,* 1875-1883.

Roth, J., Goebeler, M., van den Bos, C., and Sorg, C. (1993b) Expression of calcium-binding proteins MRP8 and MRP14 is associated with distinct monocytic differentiation pathways in HL-60 cells. *Biochem Biophys Res Commun, 191,* 565-570.

Roth, J., Teigelkamp, S., Wilke, M., Grun, L., Tummler, B., and Sorg, C. (1992) Complex pattern of the myelo-monocytic differentiation antigens MRP8 and MRP14 during chronic airway inflammation. *Immunobiology, 186,* 304-314.

Roth, J., Vogl, T., Sorg, C., and Sunderkotter, C. (2003) Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. *Trends Immunol, 24,* 155-158.

Rouleau, P., Vandal, K., Ryckman, C., Poubelle, P.E., Boivin, A., Talbot, M., and Tessier, P.A. (2003) The calcium-binding protein S100A12 induces neutrophil adhesion, migration, and release from bone marrow in mouse at concentrations similar to those found in human inflammatory arthritis. *Clin Immunol, 107,* 46-54.

Rowe, S.M., Miller, S., and Sorscher, E.J. (2005) Cystic fibrosis. *N Engl J Med, 352,* 1992-2001.

Rozmahel, R., Wilschanski, M., Matin, A., Plyte, S., Oliver, M., Auerbach, W., Moore, A., Forstner, J., Durie, P., Nadeau, J., *et al.* (1996) Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor. *Nat Genet 12,* 280-287.

Ryckman, C., Gilbert, C., de Medicis, R., Lussier, A., Vandal, K., and Tessier, P.A. (2004) Monosodium urate monohydrate crystals induce the release of the proinflammatory protein S100A8/A9 from neutrophils. *J Leukoc Biol, 76,* 433-440.

Ryckman, C., McColl, S.R., Vandal, K., de Medicis, R., Lussier, A., Poubelle, P.E., and Tessier, P.A. (2003a) Role of S100A8 and S100A9 in neutrophil recruitment in response to monosodium urate monohydrate crystals in the air-pouch model of acute gouty arthritis. *Arthritis Rheum, 48,* 2310-2320.

Ryckman, C., Vandal, K., Rouleau, P., Talbot, M., and Tessier, P.A. (2003b) Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. *J Immunol, 170,* 3233-3242.

Sade, J., Eliezer, N., Silberberg, A., and Nervo, A.C. (1970) The role of mucus in transport by cilia. *Am Rev Respir Dis, 102,* 48-52.

Saetrom, P., and Snove, O.J. (2004) A comparison of siRNA efficacy predictors. *Biochem Biophys Res Commun, 321,* 247-253.

Samuel, C.E. (2001) Antiviral actions of interferons. *Clin Microbiol Rev, 14,* 778-809.

Schafer, B.W., Fritschy, J.M., Murmann, P., Troxler, H., Durussel, I., Heizmann, C.W., and Cox, J.A. (2000) Brain S100A5 is a novel calcium-, zinc-, and copper ion-binding protein of the EF-hand superfamily. *J Biol Chem, 275,* 30623-30630.

Schafer, B.W., and Heizmann, C.W. (1996) The S100 family of EF-hand calcium-binding proteins: functions and pathology. *Trends Biochem Sci, 21,* 134-140.

Schafer, B.W., Wicki, R., Engelkamp, D., Mattei, M.G., and Heizmann, C.W. (1995) Isolation of a YAC clone covering a cluster of nine S100 genes on human chromosome 1q21: rationale for a new nomenclature of the S100 calcium-binding protein family. *Genomics, 25,* 638-643.

Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P.D. (2003) Asymmetry in the assembly of the RNAi enzyme complex. *Cell, 115,* 199-208.

Schweyer, S., Hemmerlein, B., Radzun, H.J., and Fayyazi, A. (2000) Continuous recruitment, coexpression of tumour necrosis factor-alpha and matrix metalloproteinases, and apoptosis of macrophages in gout tophi. *Virchows Arch, 437,* 534.

Schwiebert, E.M., Benos, D.J., Egan, M.E., Stutts, M.J., and Guggino, W.B. (1999) CFTR is a conductance regulator as well as a chloride channel. *Physiol Rev, 79,* S145-66.

She, Z., Wewers, M., Herzyk, D., Sagone, A., and Davis, W. (1989) Tumor necrosis factor primes neutrophils for hypochlorous acid production. *Am J Physiol, 257,* L338-345.

Sheppard, D.N., and Welsh, M.J. (1999) Structure and Function of the CFTR Chloride Channel. *Physiol Rev, 79,* S23-45.

Siegenthaler, G., Roulin, K., Chatellard-Gruaz, D., Hotz, R., Saurat, J.H., Hellman, U., and Hagens, G. (1997) A heterocomplex formed by the calcium-binding proteins MRP8 (S100A8) and MRP14 (S100A9) binds unsaturated fatty acids with high affinity. *J Biol Chem, 272,* 9371-9377.

Silva, J.M., Li, M.Z., Chang, K., Ge, W., Golding, M.C., Rickles, R.J., Siolas, D., Hu, G., Paddison, P.J., Schlabach, M.R., Sheth, N., *et al.* (2005) Second-generation shRNA libraries covering the mouse and human genomes. *Nat Genet, 37,* 1281-1288.

Sledz, C.A., Holko, M., de Veer, M.J., Silverman, R.H., and Williams, B.R. (2003) Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol, 5,* 834-839.

Smith, N.A., Singh, S.P., Wang, M.B., Stoutjesdijk, P.A., Green, A.G., and Waterhouse, P.M. (2000) Total silencing by intron-spliced hairpin RNAs. *Nature, 407,* 319-320.

Snouwaert, J.N., Brigman, K.K., Latour, A.M., Malouf, N.N., Boucher, R.C., Smithies, O., and Koller, B.H. 1992. An animal model for cystic fibrosis made by gene targeting. *Science, 257,* 1083-1088.

Song, E., Lee, S.K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar, P., and Lieberman, J. (2003) RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat Med, 9,* 347-351.

Sorensen, D., Leirdal, M., and Sioud, M. (2003) Gene silencing by systemic delivery of synthetic siRNAs in adult mice. *J Mol Biol, 327,* 761-766.

Sorg, C. (1992) The calcium-binding proteins MRP8 and MRP14 in acute and chronic inflammation. *Behring Inst Mitt, 91,* 126-137.

Sioud, M., and Sørensen, D.R. (2003) Cationic liposome-mediated delivery of siRNAs in adult mice. *Biochem Biophys Res Commun, 312,* 1220-1225.

Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., *et al.* (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature, 432,* 173-178.

Souza, L.M., Boone, T.C., Gabrilove, J., Lai, P., Zsebo, K., Murdock, D., Chazin, V.R., Bruszewski, J., Lu, H., Chen, *et al.* (1986). Recombinant human granulocyte colony-stimulating factor: effects on normal and leukemic myeloid cells. *Science, 232,* 61-65.

Steinbakk, M., Naess-Andresen, C.F., Lingaas, E., Dale, I., Brandtzaeg, P., and Fagerhol M.K. (1990) Antimicrobial actions of calcium binding leukocyte L1 protein, calprotectin. *Lancet, 336,* 763-765.

Strupat, K., Rogniaux, H., van Dorsselaer, A., Roth, J., and Vogl, T. (2000) Calcium-induced noncovalently linked tetramers of MRP8 and MRP14 are confirmed by electrospray ionization-mass analysis. *J Am Soc Mass Spectrom, 11,* 780-788.

Stutts, M.J., Canessa, C.M., Olsen, J.C., *et al.* (1995) CFTR as a cAMP-dependent regulator of sodium channels. *Science, 269,* 847-850.

Sui, G., Soohoo, C., Affar el, B., Gay, F., Shi, Y., and Forrester, W.C. (2002) A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl Acad Sci USA, 99,* 5515-5520.

Suryono, Kido, J., Hayashi, N., Kataoka, M., and Nagata, T. (2003) Effect of Porphyromonas gingivalis lipopolysaccharide, tumor necrosis factor-alpha, and interleukin-1β on calprotectin release in human monocytes. *J Periodontol, 74,* 1719-24.

Taggart, C., Coakley, R., Greally, P., Canny, G., O'Neill S.J., and McElvaney, N.G. (2000) Increased elastase by CF neutrophils is mediated by tumor necrosis factor alpha and interleukin-8. *Am J Physiol Lung Cell Mol Physiol, 278,* L33-41.

Takasaki, S., Kotani, S., and Konagaya, A. (2004) An effective method for selecting siRNA target sequences in mammalian cells. *Cell Cycle, 3,* 790-795.

Tata, F., Stanier, P., Wicking, C., Halford, S., Kruyer, H., Lench, N.J., Scambler, P.J., Hansen, C., Braman, J.C., and Williamson, R. (1991). Cloning the Mouse Homolog of the Human Cystic Fibrosis Transmembrane Conductance Regulator Gene. *Gemonics, 10,* 301-307.

Tavernarakis, N., Wang, S.L., Dorovkov, M., Ryazanov, A., and Driscoll, M. (2000) Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nat Genet, 24,* 180-183.

Taxman, D.J., Livingstone, L.R., Zhang, J., Conti, B.J., Iocca, H.A., Williams, K.L., Lich, J.D., Ting, J.P.Y., and Reed, W. (2006) Criteria for effective design, construction, and gene knockdown by shRNA vectors. *BMC Biotechnology, 6,* 7-23.

Teigelkamp, S., Bhardwaj, R.S., Roth, J., Meinardus-Hager, G., Karas, M., and Sorg, C. (1991) Calcium-dependent complex assembly of the myeloic differentiation proteins MRP-8 and MRP-14. *J Biol Chem, 266,* 13462-13467.

Terheggen-Lagro, S.W.J., Rykers, G.T., Koenderman, L., Lammers, J.W.J., and van der Ent, C.K. (2003) Neutrophil priming is increased and is related to pseudomonal load in children with cystic fibrosis. *Am J Respir Crit Care Med, 167,* A321.

Terheggen-Lagro, S.W., Rykers, G.T., and van der Ent, C.K. (2005) The role of airway epithelium and blood neutrophils in the inflammatory response in cystic fibrosis. *J Cyst Fibros Suppl, 2,* 15-23.

Thomas, G.R., Costelloe, E.A., Lunn, D.P., Stacey, K.J., Delaney, S.J., Passey, R., McGlinn, E.C., McMorran, B.J., Ahadizadeh, A., Geczy, C.L., *et al.* (2000) G551D cystic fibrosis mice exhibit abnormal regulation of inflammation in lungs and macrophages. *J Immunol,* 164, 3870-3877.

Thorey, I., Roth, J., Regenbogen, J., Halle, J., Bittner, M., Vogl, T., Kaesler, S., Bugnon, P., Reitmaier, B., Durka, S., *et al.* (2001) The Ca2+-binding proteins S100A8 and S100A9 are encoded by novel injury-regulated genes. *J Biol Chem, 276,* 35818-35825.

Tirkos, S., Chung, C., Fang, I., Kent, G., and Rozmahel, R. (2001) Investigation of S100A8 as a modifier of lung disease in CF mice. *Pediatr Pulmonol, 32,* A299.

Tirkos, S., Newbigging, S., Nguyen, V., Keet, M., Ackerley, C., Kent, G., and Rozmahel, R. (2006) Expression of S100A8 correlates with inflammatory lung disease in congenic mice deficient of the cystic fibrosis transmembrane conductor regulator. *Respir Res, 7,* 51-62.

Tiscornia, G., Singer, O., Ikawa, M., and Verma, I.M. (2003) A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc Natl Acad Sci USA, 100,* 1844-1848.

Trapnell, B.C., Chu, C.S., Paakko, P.K., Banks, T.C., Yoshimura, K., Ferrans, V.J., Chernick, M.S., and Crystal, R.G. (1991) Expression of the cystic fibrosis transmembrane conductance regulator gene in the respiratory tract of normal individuals and individuals with cystic fibrosis. *Proc Natl Acad Sci USA, 88,* 6565-6569.

Ui-Tei, K., Naito, Y., and Saigo, K. (2007) Guidelines for the selection of effective short interfering RNA sequences for functional genomics. *Methods Mol Biol, 361,* 201-216.

Ui-Tei, K., Naito, Y., Takahashi, F., Haraguchi, T., Ohki-Hamazaki, H., Juni, A., Ueda, R., and Saigo, K. (2004) Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. *Nucleic Acids Res, 32,* 936-948.

Valtieri, M., Tweardy, D.J., Caracciolo, D., Johnson, K., Mavilio, F., Altmann, S., Santoli, D., and Rovera, G. (1987) Cytokine-dependent granulocytic differentiation. Regulation of proliferative and differentiative responses in a murine progenitor cell line. *J Immunol, 138,* 3829-3835.

Vandal, K., Rouleau, P., Boivin, A., Ryckman, C., Talbot, M., and Tessier, P.A. (2003) Blockade of S100A8 and S100A9 suppresses neutrophil migration in response to lipopolysaccharide. *J Immunol, 171,* 2602-2609.

Viemann, D., Barczyk, K., Vogl, T., Fischer, U., Sunderkotter, C., Schulze-Osthoff, K., and Roth, J. (2007) MRP8/MRP14 impairs endothelial integrity and induces a caspase-dependent and -independent cell death program. *Blood, 109,* 2453-2460.

Viemann, D., Strey, A., Janning, A., Jurk, K., Klimmek, K., Vogl, T., Hirono, K., Ichida, F., Foell, D., Kehrel, B., Gerke, V., Sorg, C., and Roth, J. (2005) Myeloid-related proteins 8 and 14 induce a specific inflammatory response in human microvascular endothelial cells. *Blood, 105,* 2955-2962.

Voganatsi, A., Panyutich, A., Miyasaki, K.T., and Murthy, R.K. (2001) Mechanism of extracellular release of human neutrophil calprotectin complex. *J Leukocyte Biol, 70,* 130-134.

Vogl, T., Ludwig, S., Goebeler, M., Strey, A., Thorey, I. S., Reichelt, R., Foell, D., Gerke, V., Manitz, M.P., Nacken, W., Werner, S., Sorg, C., and Roth, J. (2004) MRP8 and MRP14 control microtubule reorganization during transendothelial migration of phagocytes. *Blood, 104,* 4260-4268.

Vogl, T., Tenbrock, K., Ludwig, S., Leukert, N., Ehrhardt, C., van Zoelen, M.A., Nacken, W., Foell, D., van der Poll, T., Sorg, C., and Roth, J. (2007) Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat Med, 13,* 1042-1049.

Wadhwa, R., Kaul, S.C., Miyagishi, M., and Taira, K. (2004) Vectors for RNA interference. *Curr Opin Mol Ther, 6,* 367-372.

Warner-Bartnicki, A. L., Murao, S., Collart, F.R., and Huberman, E. (1993) Regulated expression of the MRP8 and MRP14 genes in human promyelocyticleukemic HL-60 cells treated with the differentiation-inducing agents mycophenolic acid and 1 alpha, 25-dihydroxyvitamin D3. *Exp Cell Res, 204,* 241-246.

Waterhouse, P.M., Wang, M.B., and Lough, T. (2001) Gene silencing as an adaptive defence against viruses. *Nature, 411,* 834-842.

Weber, A.J., Soong, G., Bryan, R., Saba, S., and Prince, A. (2001) Activation of NF-nB and altered InB-h processing in cystic fibrosis bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol, 281,* L71-78.

Welsh, M.J. (1987) Electrolyte transport by airway epithelia. *Physiol Rev, 67,* 1143-1183.

Welsh, M.J., and Smith, A.E. (1995) Cystic Fibrosis. *Sci Am, 273,* 52-59.

Welsh, M.J., and Smith, A.E. (1993) Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell, 73,* 1251-1254.

Wilkinson, M.M., Busuttil, A., Hayward, C., Brock, D.J., Dorin, J.R., and Van Heyningen, V. (1988) Expression pattern of two related cystic fibrosis-associated calcium-binding proteins in normal and abnormal tissues. *J Cell Sci, 91,* 221-230.

Williams, B.R. (1997) Role of the double-stranded RNA-activated protein kinase (PKR) in cell regulation. *Biochem Soc Trans, 25,* 509-513.

Winter, J., Jung, S., Keller, S., Gregory, R.I., and Diederich, S. (2009) Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nature Cell Biology, 11,* 228-234.

Xia, G., Kumar, S.R., Masood, R., Zhu, S., Reddy, R., Krasnoperov, V., Quinn, D.I., Henshall, S.M., Sutherland, R.L., Pinski, J.K., *et al.* (2005) EphB4 expression and biological significance in prostate cancer. *Cancer Res, 65,* 4623-4632.

Xu, K., and Geczy, C.L. (2000) IFN-gamma and TNF regulate macrophage expression of the chemotactic S100 protein S100A8. *J Immunol, 164,* 4916-4923.

Xu, K., Yen, T., and Geczy, C.L. (2001) Il-10 up-regulates macrophage expression of the S100 protein S100A8. *J Immunol, 166,* 6358-6366.

Xu, P., Guo, M., and Hay, B. A. (2004) MicroRNAs and the regulation of cell death. *Trends Genet, 20,* 617-624.

Yang, Z., Tao, T., Raftery, M.J., Youssef, P., Di Girolamo, N., and Geczy, C.L. (2001) Proinflammatory properties of the human S100 protein S100A12. *J Leukoc Biol, 69,* 986-994.

Yen, T., Harrison, C.A., Devery, J.M., Leong, S., Ismaa, S.E., Yoshimura, T., and Geczy, C.L. (1997) Induction of the S100 chemotactic protein, CP-10, in murine microvascular endothelial cells by proinflammatory stimuli. *Blood, 90,* 4812-4821.

Yi, R, Doehle, B.P., Qin, Y., Macara, I.G., and Cullen, B.R. (2005) Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs. *RNA, 11,* 220-226.

Yi, R., Qin, Y., Macara, I.G., and Cullen, B.R. (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev, 17,* 3011-3016.

Yi, Y., Singh, A., Cutilli, J., and Collman, R.G. (2004) Use of dual recombinant vaccinia virus vectors to assay viral glycoprotein-mediated fusion with transfection-resistant primary cell targets. *Methods Mol Biol, 269,* 333-346.

Young, F.D., Choi, C., Newbigging, S., Keet, M., and Rozmahel, R. (manuscript in preparation) Rescue of cystic fibrosis intestinal disease in mice by mCLCA3.

Yu, J., DeRuiter, S., and Turner, D. (2002) RNA Interference by Expression of Short-interfering RNAs and Hairpin RNAs in Mammalian Cells. *Proc Nat Acad Sci, 99,* 6047-6052.

Yu, J., Taylor, J., DeRuiter, S., Vojtek, A., and Turner, D. (2003) Simultaneous inhibition of GSK3-α and GSK3-β using hairpin siRNA expression vectors. *Molec Ther, 7,* 228-236.

Yui, S., Mikami, M., and Yamazaki, M. (1995) Purification of the cytotoxic factor in rat peritoneal exudate cells: its identification as the calcium binding protein complex, calprotectin. *J Leukoc Biol, 58,* 307-316.

Zabner, J., Fasbender, A.J., Moninger, T., Poellinger, K.A., and Welsh, M.J. (1995) Cellular and Molecular barriers to Gene Transfer by Cationic Lipid. *The Journal of Biological Chemistry, 270,* 18997-19007.

Zhang, G., Budker, V., and Wolff, J.A. (1999) High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum Gene Therap, 10,* 1735-1737.

Zhang, H., Kolb, F.A., Brondani, V., Billy, E., and Filipowicz, W. (2002) Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. *EMBO J, 21,* 5875-5885.

Zimmer, D.B., Cornwall, E.H., Landar, A., and Song, W. (1995) The S100 protein family: history, function, and expression. *Brain Res Bull, 37,* 417-429.

Zimmer, D.B., Wright, R., Sadosky, P., and Weber, D.J. (2003) Molecular mechanisms of S100- target protein interactions. *Microsc Res Tech, 60,* 552-559.

Zimmermann T.S., Lee A.C., Akinc A., Bramlage B., Bumcrot, D., Fedoruk M.N., Harborth J., Heyes J.A., Jeffs L.B., *et al.* (2006) RNAi-mediated gene silencing in non-human primates. *Nature, 441,* 111-114.

Zwadlo, G., Bruggen, J., Gerhards, G., Schlegel, R., and Sorg, C. (1988) Two calcium-binding proteins associated with specific stages of myeloid cell differentiation are expressed by subsets of macrophages in inflammatory tissues. *Clin Exp Immunol, 72,* 510-515.

Zwadlo, G., Schlegel R., and Sorg, C. (1986) A monoclonal antibody to a subset of human monocytes found only in the peripheral blood and inflammatory tissues. *J Immunol, 137,* 512-518.