

SYMPOSIUM SUMMARIES

S1.2

REGULATION OF CFTR ACTIVITY VIA PROTEIN-PROTEIN INTERACTIONS

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The ATP-binding cassette (ABC) superfamily proteins are important functional transporters in both prokaryotes and eukaryotes, playing primary roles in mediating the entry and exit of a variety of molecules. The CFTR protein is a member of the ABC transporters. The interactions of the CFTR with other structural and regulatory proteins are essential for the proper function of the CFTR in health and disease.

Protein-protein interactions are intrinsic to virtually every cellular process. At the biochemical level, protein-protein interactions have been found in multi-subunit proteins, assembled functional protein complexes, and transient protein-protein contacts. Consequently, these interactions lead to a broad spectrum of biological outcomes that include alteration of kinetic properties and/or activity of an enzyme, substrate channeling, and formation of a new binding site and/or changes of its substrate. Because the degree of regulation that protein-protein interactions confer is large, investigation of their physiological implications requires (i) identification of the different interactions and the molecular components, (ii) determination of the extent to which they take place in the cell, and (iii) determination of the consequences of the interaction. Recent progress in this area has revealed a class of very interesting proteins whose function is dedicated to the spatial and stoichiometric organization of various signaling and transport proteins. The primary structure of these scaffold proteins usually contains multiple protein interaction modules; each often interacts with a given target protein. As a result, they are capable of recruiting and organizing protein machinery with defined composition and stoichiometry, thereby providing high specificity and efficiency to the corresponding biochemical reactions such as ion transport and signal transduction.

Using molecular and biochemical approaches, a number of scaffolding proteins have been identified on the basis of their ability to interact with the C-terminus of the CFTR. These scaffolding proteins include CAL (CFTR-Associated Ligand), NHE-RF (Na⁺-H⁺ Exchanger Regulatory Factor) and CAP70 (CFTR-Associated Proteins 70Kd). In addition to their potential role in trafficking the interaction proteins, purified recombinant proteins of CAP70 and NHE-RF have been shown to directly potentiate the chloride channel activity of CFTR via a stoichiometric interaction that is consistent with the inductive formation of a CFTR dimer by CAP70 or NHE-RF. This evidence supports the notion that in addition to the conventional role of PDZ domain in spatial positioning of binding protein to specific subcellular locations or to other functionally coupled proteins, PDZ domain containing proteins may also play a role in regulating functionality of their target proteins by defining the stoichiometry and geometry of a functional unit.

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S1.3

CFTR REGULATION BY INTERMOLECULAR AND INTRAMOLECULAR INTERACTIONS

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The opposing cytoplasmic tails of CFTR are engaged in distinct sets of protein-protein interactions. PDZ domain-mediated interactions with the carboxy terminal tail of CFTR can modulate the intracellular location and

functional activities of CFTR channels. We have determined that the amino terminal tail (N-tail) also participates in intermolecular and intramolecular interactions that govern CFTR channel activity. Three topics will be

addressed in my presentation: (i) interactions between the CFTR N-tail and SNARE components of the membrane traffic machinery; (ii) the participation of the N-tail in an intramolecular interaction that modulates channel gating and (iii) interactions between CFTR channels and the p11-Annexin II complex.

CFTR-SNARE interactions SNAREs mediate membrane fusion in eukaryotic cells by forming protein complexes that consist of two SNAREs at the target membrane (t-SNAREs) and one SNARE on the vesicle membrane (v-SNARE). A subset of t-SNAREs can also bind to and regulate certain ion channels (1,2). For example, CFTR channels physically and functionally interact with two plasma membrane-associated t-SNAREs, syntaxin 1A (3-4) and SNAP-23 (unpublished data). Both t-SNAREs bind to the CFTR N-tail and inhibit CFTR-mediated chloride currents in multiple expression systems and epithelial cell types. The regulation of CFTR by syntaxin 1A is isoform-specific; namely, syntaxins 2-5 cannot physically or functionally interact with CFTR. Interestingly, syntaxin 1A and SNAP-23 display apparent cooperativity with respect to binding to and regulating CFTR. This implies that CFTR interacts more robustly with the t-SNARE heterodimer (i.e., the syntaxin 1A-SNAP-23 dimer) than with either SNARE alone.

What is the physiologic role of interactions between CFTR channels and SNAREs? Conceivably, the exocytic insertion or endocytic retrieval of CFTR channels at the plasma membrane could be facilitated by physical interactions with t-SNAREs (5). In addition, SNAREs may directly regulate CFTR channel gating as a means to couple the activity of this ion channel to alterations in membrane traffic in epithelial cells. We favor a model in which t-SNAREs modulate CFTR channel gating via direct protein-protein interactions on the basis of two sets of observations. First, we have discovered that the CFTR N-tail (to which both t-SNAREs bind) plays a significant role in modulating CFTR channel gating (see below). Second, we have generated point mutants of syntaxin 1A that are defective for binding CFTR and for regulating CFTR currents, but that are unaffected for SNARE complex assembly. Thus, it appears that we can dissociate the effects of syntaxin 1A on CFTR channel activity from the participation of this t-SNARE in membrane traffic. This latter observation raises the possibility that the regulation of ion channels by certain SNAREs is a distinct functional property that is independent of their involvement in membrane fusion reactions. In this regard, it is becoming increasingly clear that syntaxin 1A can bind to and regulate multiple types of ion channels and transporters (e.g., synaptic calcium channels (1), CFTR (3-5), ENaC (6) and neurotransmitter transporters (7)). In each case the effect of syntaxin 1A is to inhibit transport. Perhaps specific SNAREs such as syntaxin 1A and SNAP-23 can limit the permeabilities of cells to a variety of solutes to protect against changes in cell com-

position and volume under certain conditions (e.g., during osmotic or hypoxic stress). Interactions between SNAREs and ion channels appear to constitute a general paradigm in transport biology that includes but is not limited to CFTR.

Involvement of the N-tail in CFTR channel gating Our functional analysis of the CFTR N-tail has led to the identification of a helical subdomain (residues 46-60) that modulates channel gating. Mutating or chemically modifying a 'stripe' of acidic residues along one surface of this helix reduces channel activity by affecting both the opening and closing of the channel (ref.8 and unpublished data). Using a peptide mapping strategy in combination with deletion analysis we have identified a putative docking site for the N-tail 'gating helix' that resides between residues 595 and 623. Until recently this region was thought to reside within the proximal portion of the R domain; however, it is now apparent that residues 595-623 represent the distal portion of NBD1 (9). In fact, based on recent structural studies of the NBD of the bacterial maltose transporter (MalK (10)), it appears likely that this region represents a 'switch domain' that couples nucleotide binding and hydrolysis at the NBD to the gating of the translocation pathway (i.e., pore). This raises the interesting possibility that the N-tail is a component or a regulator of the effector mechanism that links ATP binding at NBD1 to channel opening and closing. I will summarize the results of recent biochemical and functional studies that are consistent with such a model.

Interactions between CFTR and the p11-annexin II complex. The annexin II-p11 complex is also capable of interacting with the CFTR N-tail. Annexin II and p11 form a heterotetrameric complex that participates in several signaling pathways that are potentially relevant to CFTR biology (e.g., cPLA₂ signaling). We identified annexin II as an N-tail binding protein by mass spectroscopic analysis of GST-N-tail binding proteins purified from epithelial cell extracts. Further analysis revealed that this interaction requires the presence of p11, and that p11 binds directly to the CFTR N-tail in pairwise binding assays. Purified recombinant p11 stimulates CFTR channel activity by 30-80% in inside-out patches excised from CFTR-expressing BHK cells (EC₅₀ of 300-400 nM). Thus, this interaction appears to be functionally relevant. At present we do not know the relationship, if any, between this interaction with the CFTR N-tail and the regulation of CFTR by SNAREs. However, these recent data do indicate that the amino terminal tail of CFTR is capable of linking this ion channel to multiple and diverse regulatory pathways. The p11-annexin II interaction in particular could couple CFTR activity to signaling pathways that involve this protein complex.

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S1.4 CRYSTALLOGRAPHIC STUDIES OF NUCLEOTIDE-BINDING DOMAINS FROM ABC TRANSPORTERS: IMPLICATIONS FOR THE MECHANOCHEMISTRY OF CFTR

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Although the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) functions as an ATP-activated anion channel, it is strongly homologous to a superfamily of ATP-powered transmembrane solute pumps called "ATP-Binding Cassette Transporters" (abbreviated ABC Transporters). During the last two years, crystal structures have been determined for the nucleotide-binding domains (NBD's) from several ABC transporters in nucleotide-free, ATP-bound, and ADP-bound conformations. These structures allow inferences to be drawn concerning the mechanochemical reaction cycle of the homologous NBD's in CFTR, *i.e.* the conformational changes that drive channel opening and closing in response to sequential ATP-binding, hydrolysis, and release.

The existing crystal structures indicate that a modest induced-fit conformational change occurs upon nucleotide binding, involving reorientation and/or dynamic stabilization of the active site loops that contact the nucleotide. The rotational orientation of the alpha-helical subdomain of the NBD appears to be controlled by the presence of the gamma-phosphate of ATP in the active site based primarily on the formation of a hydrogen bond between the gamma-phosphate and a phylogenetically-invariant glutamine located at the end of a linker peptide that connects the alpha-helical subdomain to the

ATP-binding core of the NBD. On this basis, ATP binding is inferred to cause a large rotation of the alpha-helical subdomain of the NBD, leading to approximately a 10 angstrom movement of the "LSGGQ" transporter signature sequence that is located in that subdomain, and this rotation is likely to be reversed upon ATP hydrolysis prior to ADP release.

However, mutation of the invariant glutamine leads to only a modest functional defect in a model ABC Transporter, making it unlikely that the gamma-phosphate-dependent rotation of the alpha-helical subdomain represents the power-stroke of the pump. Mutation of the equivalent glutamine in NBD1 of CFTR likewise produces only modest changes in gating behavior. These results indicate that the power-stroke of the ABC Transporters as well as the gating event in CFTR are likely to be driven by ATP-dependent changes in NBD-NBD interactions rather than by the nucleotide-dependent conformational changes in the individual NBD's.

Thus, understanding the nature of the interactions between the NBD's in CFTR is likely to be critical to understanding its molecular mechanism and the regulation of its gating behavior. However, it is unknown whether NBD1 interacts with NBD2 or whether each of the NBD's forms an independent homodimer in intact CFTR. The latter arrangement of tandem NBD homodimers is

only possible if CFTR itself forms a homodimer in the membrane, making resolution of the current controversy concerning the oligomeric structure of CFTR of the utmost importance in understanding its mechanism. The data both favoring and opposing dimerization of CFTR will be reviewed, with an emphasis on the impor-

tance of this issue in formulating and interpreting detailed molecular models of ATP-dependent channel gating. Finally, the location of common disease-causing mutations will also be reviewed with regard to their possible effect on the mechanochemistry and inter-NBD interactions in CFTR.

S2.1

INTERACTION OF ANTIGEN PRESENTING CELLS WITH *PSEUDOMONAS AERUGINOSA*

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Infection of the respiratory tract with *Pseudomonas aeruginosa* (PA) is a hallmark of the lung disease associated with CF and is a major cause of morbidity and mortality. The mechanism by which PA can establish infection in the lung are complex and linked in part to the ability of the PA population to develop biofilms, and in part by the capacity of PA to interfere with the host defense mechanisms arrayed against pathogens. Critical to the pulmonary defense against extracellular bacteria is the sampling and processing of the bacteria by antigen presenting cells, including alveolar macrophages (AM) and dendritic cells (DC). AM play a central role in clearing extracellular bacteria and DC are the most potent antigen presenting cells and are present in the lung interspersed the airway epithelial cells and in the lung parenchyma. We have previously shown that immunization with murine bone marrow derived DC pulsed PA can induce protection against a subsequent lethal pulmonary infection with agar encapsulated PA in a mouse model. Studying the interaction of antigen presenting cells with PA and using genetic modification of these cells could be useful in the development of new therapies against PA infection in CF. As several recent studies have shown that PA can be cytotoxic, and can induce apoptosis in a variety of cell types, one focus of the present study is to analyze the ability of PA to induce programmed cell death in antigen presenting cells.

AM and DC were susceptible to cell death induced by the laboratory PA isolates PAO1, PAK and PA103 as well as a variety of PA isolates derived from sputum of individuals with CF. Apoptosis, analyzed by TUNEL assay, was detectable in AM and DC as early as 3 hr after infection with PA. In contrast, the same strains and doses of PA had little effect on the lung epithelial cell line A549 and human bronchial epithelial cells *in vitro*. Pre-treatment of DC with the caspase inhibitors ZVAD-fmk and YVAD-cmk reduced PA-induced cell death ($p < 0.05$). Genetic modification of DC to express CD40L, a CD4⁺ T cell molecule that plays a central

role in activating antigen presenting cells, using an adenovirus vector decreased the susceptibility of DC to cell death induced by PAO1 compared to DC infected with a control Ad vector ($p < 0.01$). This data demonstrates that DC and AM are susceptible to apoptosis induced by PA and that this response can be partially reversed by genetic modification with CD40L. These observations suggest a potential mechanism contributing to the persistence of PA in CF and suggest that genetic manipulation of antigen presenting cells with anti-apoptotic genes may be able to strengthen host defenses in CF.

To explore the mechanisms of the genetically modified DC in their interaction with PA further, current studies are directed at the analysis of the cellular responses of naive or genetically modified DC to PA using microarray technology. Identifying pathways and factors following exposure of DC to PA or its components may help to identify new target for genetic modification. Analysis of the transcript profiles in murine bone marrow derived DC following exposure to the laboratory PA strain PAK (live and heat inactivated) and the pseudomonas component LPS demonstrated 2-fold differential expression of more the 200 genes compared to unstimulated control cells. Analysis of the profile clusters of transcripts upregulated following exposure to live, heat-killed PA and LPS contained cytokines and chemokines expected to be activated following bacterial contact. The largest cluster of genes was found following exposure to live and heat inactivated PA but not in response to LPS. Transcripts upregulated following exposure to the live bacteria included transcription factors and genes related to apoptosis. Interestingly, a cluster of 28 genes could be identified following exposure to heat-inactivated PA, but not in the other groups, containing a variety of genes related to transcription and adhesion of the cells. Additional studies analyze the response of human AM to different PA strains including clinical isolates derived from an individual with CF obtained at different stages of the disease and *Burkholderia cepacia*

(BC) using a human microarray. Infection of AM with PAK for three hours resulted in more than 2 fold higher transcript levels for 183 genes compared to uninfected controls, including a variety of genes unknown to be associated with macrophage activation or infection. Similar total numbers of genes being upregulated more than 2 fold were found following exposure to an early and late clinical isolate and to BC. Genes for cytokines such as IL-6 and TNF were upregulated similarly comparing the different strains. However differences between the early and late clinical isolate were observed with genes related to apoptosis and transcription activation. Dis-

tinct differences were also seen in these categories following exposure to BC.

This data suggests that the different transcript profiles observed in response to different bacterial isolates may identify common pathways of cellular responses to the bacterial exposure. The analysis of activation and response pathways may lead to the identification of new targets for genetic modification, and aid in the development of strategies to eliminate PA from the respiratory tract.

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S2.3

MNEI: NOVEL ANTI-PROTEASE THERAPY FOR CF LUNG DISEASE

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Lung disease is the major cause of morbidity and mortality in cystic fibrosis (CF) and is characterized by intense neutrophil influx. Although debate continues about the chronology, there is general agreement that neutrophil dominated inflammation and chronic infection are major causes of loss of airway function.

In the chronically inflamed lungs of CF patients, the activities of the neutrophil's abundant serine proteases, elastase, cathepsin G and proteinase-3, are turned upon lung tissue. Elastase, the best characterized, has broad substrate specificity, degrading elastin and other structural proteins, impairing the anti-bacterial functions of macrophages and neutrophils by targeting components of opsonophagocytosis pathways, enhancing mucin release from tracheal epithelial cells and stimulating production of proinflammatory cytokines including IL-8. Although the cleavage specificities of the three proteases differ, cathepsin-G and proteinase-3 cleave many of the same targets as elastase or show synergetic effects.

It is well established that the normal antiprotease system is overwhelmed in CF leading to unopposed neutrophil proteolytic activity, which suggests that boosting airway antiprotease capacity has the potential to slow the decline of lung function. MNEI (monocyte/neutrophil elastase inhibitor), a naturally occurring 42 kilodalton human protein of known sequence appears to be a promising therapeutic. In vitro and animals studies suggest that MNEI, if delivered as an aerosol to airways of CF patients, will restore the antiprotease barrier and interfere in protease-mediated pathological events. MNEI is a serpin (SERine Protease INhibitor) molecule, a member of the superfamily that includes alpha-1-antitrypsin and 30 other human proteins and glycoproteins. It functions by the mechanism unique to serpins, binding and simultaneously inactivating protease by forming a 1:1 covalent complex.

Determination of rate constants and stoichiometry indicate that MNEI is an efficient inhibitor of two classes of serine proteases, those with elastase-like activity including neutrophil elastase and proteinase-3 and those with chymotrypsin-like activity including cathepsin-G. MNEI does not inhibit trypsin-like proteases, plasmin, thrombin, plasminogen activator. The dual specificity of MNEI was shown to derive from the presence of two reactive sites, one with an interacting cysteine residue to inhibit elastase and proteinase-3 and an adjacent site with an interacting phenylalanine residue to inhibit cathepsin G. The reactions of MNEI are particularly rapid; rate constants of inhibition are 3.4×10^7 , 1.7×10^7 , and $2.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ for elastase, proteinase-3 and cathepsin G, respectively. MNEI, which is produced by monocytes and neutrophils and released by mechanisms that have not yet been defined, is present at high levels ($> 5 \text{ g/ml}$) in bronchoalveolar (BAL) fluids of patients with CF and is non-detectable or at low levels in airway fluids of non-CF patients. MNEI in BAL of CF patients is partially found in complex with protease, but mainly as the inactivated "post-complex" species, likely the result of the overwhelming levels of elastase. Importantly, addition of active recombinant MNEI to elastase-containing CF airway secretions (sputum sol) causes dose-dependent and complete inhibition of elastase activity, strongly suggesting that recombinant MNEI, if administered at adequate levels, will function in the milieu of the inflamed CF airway.

When evaluated in a rat model of elastase-induced acute lung damage, recombinant MNEI protected against injury (quantified as hemorrhage) and prevented the increase of epithelial permeability due to instillation of neutrophil elastase or elastase-containing airway secretions from CF patients. When rats were instilled with fluorescently labeled MNEI, the signal was detectable on large airways up to 24 hours later. Delivery of MNEI

up to 24 hours before challenge with elastase protected the rats from lung injury.

Recombinant MNEI is currently being studied in an animal lung infection model that replicates many pathological features of CF lung disease. In the model, *Pseudomonas aeruginosa* in agar beads is deposited intratracheally in rat lungs. Inoculation leads to chronic infection, inflammation and lung damage, which can be quantified as a histopathology score (1). Daily aerosol treatment with recombinant MNEI beginning either 2 or 7 days post-inoculation with *Pseudomonas* significantly decreased the histopathology score compared to inoculated animals treated only with saline. Importantly, daily aerosol delivery of rMNEI significantly decreased bacterial burden compared to inoculated animals that received only saline. Similar decrease of the *Pseudomonas* burden was previously achieved in this system by delivery of alpha-1-antitrypsin (Prolastin[®]) (2). Since MNEI has no intrinsic anti-bacterial activity, its ability to enhance clearance of *Pseudomonas* suggest that MNEI protects or enhances innate anti-bacterial defense mechanisms of rat lungs.

The next phase of evaluating MNEI involves large-scale production and formulation, efficacy and stability studies, toxicology and pharmacokinetic studies in animals in preparation for clinical trials in patients. While continuing to study insect cell derived recombinant MNEI, we established an alternate system suitable for large-scale production by generating MNEI-expressing recombinants of the yeast *Pichia pastoris*. Recombinant *Pichia* were grown and analyzed initially in small flasks and most recently in 5-liter fermentors. Advantages of the *Pichia* system include economy, ease of scaleup, and the use of only simple defined media without animal-derived raw materials. Since naturally occurring human MNEI is a non-glycosylated protein, the *Pichia* system was engineered for intracellular production, thus avoiding yeast derived glycosylation. Production

levels are in the grams per liter range. Purification protocols are in development based in part on chromatography established for MNEI isolation from monocytes and insect cells.

Collectively, the following evidence supports the potential efficacy as well as practicability of developing recombinant MNEI as a therapeutic for CF:

Novel Properties of rMNEI Represent Advantages as an Anti-Protease Therapy for CF

- Spectrum of antiprotease activity includes all three major neutrophil proteases
- Acts exclusively on extracellular proteases
- Spectrum of activity excludes proteases of coagulation and complement systems
- Blocks damage induced in rat lung injury model by CF sol
- Enhances *Pseudomonas* clearance and decreases injury in chronically infected rat lungs
- Stable following lyophilization (potential for delivery as a liquid or dry powder)
- Long-lasting bioavailability in the (rat) airway
- Non-glycosylated (likely to be non-immunogenic)
- Produced economically in *Pichia* system

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S2.4

ANTI-INFLAMMATORY MONOCLONAL ANTIBODY THERAPEUTIC AGENTS

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The use of antibodies for therapy began in 1890 when Von Behring and Kitasato discovered antidipteria toxins.¹ Over the years hyperimmune serum from patients has been used to treat infections such as rabies and tetanus. In the past two decades, rapid advances in genetic engineering have made it possible to enhance the specificity of the antibody combining sites, to alter its size, structure, and shape, and thus to increase the therapeutic utility of antibody

ies while minimizing their immunogenicity.² Twenty-five years after their advent, monoclonal antibodies (MAb) have emerged as an important and rapidly expanding new drug class for the treatment of severe human diseases.^{3,4} Ten antibodies have been approved in the US for diverse clinical indications: cancer, transplant rejection, Crohn's disease, respiratory syncytial virus (RSV) prophylaxis and as an anti-thrombotic (Table 1). An additional 70 or so antibody

therapeutics are in clinical trials in the US including 42 that have progressed to phase I/II or further. Numerous difficulties hampering the development of antibody therapeutics have long been recognized and extensively reviewed.^{5,6} These problems with MAb drugs are related to the choice of antibody, antigen and target tissue but also include pharmaceutical and clinical issues.

By far the most important class of antibody therapeutics is immunoglobulin G (IgG) and derived fragments, with only a very few IgM and IgA molecules currently in clinical development. IgGs are tetrameric, Y-shaped molecules (~150 kDa) that contain two antigen-binding sites. Antigen binding is mediated primarily by six loops, known as complementarity-determining regions (CDRs), three of which are contributed by each of the V_H and V_L domains. A few core antibody technologies have been crucially important to the development of all marketed antibody therapeutics as well as to virtually all antibody therapeutics in clinical development. These core antibody technologies are: hybridoma, chimerization and human antibodies through phage libraries or transgenic mice. Humanized and human antibodies are the currently preferred core technologies for developing antibody therapeutics. Humanization may be the favored strategy when a well-characterized murine MAb is available. In contrast, for a newly identified antigen where no MAbs are available, direct production of human antibodies using mice or phage are preferred. Many more human antibodies from phage and mice are anticipated to enter clinical trials as these technologies become more widely available. The production of protein pharmaceuticals in the milk of transgenic animals is a promising technology that has yet to gain widespread acceptance and use. IgG molecules have been produced in the milk of transgenic goats at titers of up to 14g/L. A significant downside to the production of protein pharmaceuticals in transgenic animals is that animal breeding is likely to add several years to the development timeline as com-

pared to traditional mammalian cell culture technology. Recent advances in whole animal cloning,⁷ including the production of a human protein⁸ have the potential to partially offset this timeline penalty.

Anti-Inflammatory antibodies represent an important new class of drugs whose impact will doubtless increase as molecules in clinical trials (Table 2) progress through to FDA approval.

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Table 1. Currently Approved Antibody Therapeutics

OKT3	Murine	Transplant rej.	1988	OrthBiotech
ReoPro	Chim.Fab	Cardiovascular	1994	Centocor/Lilly
Panorex	Murine	Colorectal cancer	1995*	Centocor/Glaxo
Rituxan	Chimeric	NHL	1997	IDEC/Genentech
Zenapax	Humanized	Transplant rej.	1998	Roche
Simulect	Chimeric	Transplant rej.	1998	Novartis
Remicade	Chimeric	Crohn's disease	1998	Centocor
Herceptin	Humanized	Breast cancer	1998	Genentech
Synagis	Humanized	RSV infection	1998	MedImmune
Mylotarg	Humanized	AML	2000	Wyet

Table 2. Antibody-Derived Anti-inflammatory Therapeutic Agents in Clinical Trials in the United States

Antibody Names	Target	Proposed Use	Sponsor
ABX-IL8	IL8	Psoriasis	Abgenix
RhuMab-E25	IgE	Allergic rhinitis, allergic asthma	Genentech, Novartis
LeukArrest Hu23F2G	CD11/CD18	Myocardial infarction, multiple sclerosis	ICOS
CDP571	TNF- ∞	Crohn's disease, rheumatoid arthritis, inflammatory bowel disease	Celltech
Anti-CD11a	CD11a, $\infty_4\beta_7$ integrin	Psoriasis	Genentech, Xoma
LDP-02	Integrin	Ulcerative colitis	LeukoSite
LDP-01	Leukocyte, β_2 integrin	Stroke, kidney	LeukoSite
LymphoCide H5G1.1	CD22	Non-Hodgkin's lymphoma Systemic lupus, erythematosus, Rheumatoid arthritis	Immunomedics, Alexion Pharmaceuticals
Primatized-IDEA-151, IDEA-CE9.1, C225	CD4	Rheumatoid arthritis	IDEA Pharmaceuticals, Smith Kline Beecham
Anti-LFA1 odulimomab	CD18	Transplant rejection	Pasteur-Merieux, Immunotech
MAK-195F, Segard afelimomab	TNF- ∞	Septic shock	Knoll Pharmaceutical, BASF
ABX-CBL	Activated B and T cells, monocytes	Transplant rejection	Abgenix
HNK20	RSV, F glycoprotein	RSV infection	Peptide Therapeutics Group

S3.1

THE ROLE OF TYPE III TOXINS IN PSEUDOMONAS PATHOGENESIS

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Pseudomonas aeruginosa is an opportunistic pathogen that can cause a variety of infections in compromised individuals. Acute infections include those associated with colonization of the urinary tract, burn wounds, corneal epithelium, otitis media, and nosocomial pneumonia. *P. aeruginosa* is the most frequent Gram-negative bacterium involved in nosocomial pneumonia. Acute pneumonias associated with *P. aeruginosa* have a high mortality rate despite appropriate antibiotic treatment. Cystic fibrosis (CF) patients become chronically colonized with *P. aeruginosa*. These infections remain localized to the lung and do not generally result in disseminated disease. Chronic infections, however, appear to lead to intense, sustained host inflammatory responses, which contribute to lung deterioration and mortality.

In addition to the differences in clinical outcome between acute and chronic infections, *P. aeruginosa* isolated from chronically infected individuals differ from those obtained from individuals suffering acute infections. Documented changes in strains isolated from chronically infected individuals include the acquisition of mutations in genes regulating alginate synthesis, loss of flagellar gene expression, the loss of O antigen expression, and a general down regulation in the expression of most of the classical virulence determinants (1,2,3) consistent with the mucoid phenotype. The mechanisms mediating the conversion to mucoidy are postulated to involve selection pressure in the airway of CF patients and or colonization by hypermutator strains (4,5). Recent evidence suggests that CF patients are colonized intermittently during their first three years of life (6). Most of these early isolates are non mucoid and express some of the classical virulence associated factors (6). It is unclear whether these initial infections lead to epithelial injury or program inappropriate responses that enhance the establishment of the chronic form of the organism. Thus preventing early infections may be a key strategy to preventing or delaying subsequent chronic infection (7).

P. aeruginosa is a well-studied organism whose virulence is associated with the expression of an arsenal of factors that contribute to pathogenesis. The first step in infection, adhesion to tissue, appears to be mediated by several factors that include flagella, lipopolysaccharide, and pilin. *P. aeruginosa* produces and secretes a number of enzymes that are associated with virulence, including the phospholipases (hemolytic and non-hemolytic), neuraminidase, proteases, enzymes involved in nucleotide metabolism, and ADP-ribosyltransferases. Most of these activities are associated with tissue destruction and host cell death. The architecture of the bacterial outer surface and the ability to grow in biofilms is likely essential for full virulence in chronic infections. While many virulence associated factors have been identified and genetically characterized there are significant gaps in our understanding of how these factors are regulated, when they are expressed, and how their expression may change over time or in response to the host environment in chronically infected individuals.

Our studies have focused on the expression of the type III secretory system of *P. aeruginosa* as a model for events that occur early in acute infections and during the intermittent phase preceding establishment of chronic infection (6,8). The type III secretion system consists of three separate but coordinately functional protein complexes: the secretion apparatus, the translocation or targeting complex, and the secreted toxins and cognate chaperones (9). Four known toxins are injected by *P. aeruginosa*, ExoS, ExoT, ExoY, and ExoU (10). ExoS and ExoT possess Rho GAP activity and are ADP-ribosyltransferase enzymes (11). Both ExoS and ExoT cause profound changes in the cellular cytoskeleton in vitro (12) and likely prevent phagocytosis and wound healing in vivo (13). ExoY is an adenylate cyclase that also causes cytoskeletal changes through the generation of high intracellular levels of cAMP (14). ExoU and ExoS are cytotoxic (15,16,17). The cytotoxicity of ExoS is mediated by its ability to ADP-ribosylate members of the Ras superfamily of proteins that control essential cellular processes (11,15). ExoU is a potent cytotoxin but the mechanism of action is unclear. Structure and functional studies of the cellular targets for the type III toxins are ongoing using mammalian cells and yeast as model systems. In addition, the inhibition of toxin translocation appears to prevent *Pseudomonas*-induced lung injury and greatly increase survival in acute lung infections (18). The development of vaccines against specific type III components may be a viable strategy for preventing initial infections in young CF patients (8).

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S3.2

GENETIC AND GENOMIC DISSECTION OF HOST-PATHOGEN INTERACTIONS USING A *P. AERUGINOSA* - *C. ELEGANS* PATHOGENESIS MODEL

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Host-pathogen interactions are antagonistic relationships, in which the success of each organism depends on its ability to overcome the other. As a result of these interactions, host organisms have developed surveillance systems to detect, and an arsenal of effector molecules to destroy, invading microbes. Likewise, pathogens have evolved strategies to evade or overcome these defenses. Despite great advances made in improving public hygiene, and in the discovery and use of antimicrobials and vaccines, infectious agents remain the main cause of mortality worldwide. *P. aeruginosa* is one of the most common pathogen associated with primary blood stream infections and pneumonia in pediatrics intensive care units (Richards et al., 1999) and an important pathogen in immunocompromised and Cystic Fibrosis (CF) patients. Efforts to counter the threat by this pathogen require a better understanding of the cellular and molecular mechanisms underlying the interactions between the pathogen and its host. Fundamental questions need to be addressed: What are the virulence factors employed by *P. aeruginosa* to overcome host defenses? What are the signaling pathways that are triggered, and the defense arsenal that is deployed, by the host in response to pathogens? What are the molecular mechanisms underlying the interaction between host- and pathogen-derived factors? In the following, we describe a *P. aeruginosa* - *C. elegans* pathogenesis model system that is amenable to combining robust genetic, genomic and molecular approaches in a single experimental system to approach these questions. Because *C. elegans* and *P. aeruginosa* are amenable to genetic analysis, both the host and pathogen can be genetically altered and the effects of these alterations on pathogenesis can be tested. The availability of complete genome sequences of both the host and pathogen also provides an opportunity to use genomic approaches, such as DNA chip technology, to

perform genome-wide gene expression analyses on the host and pathogen as they interact.

P. aeruginosa kills the nematode *C. elegans* by at least 3 largely distinct mechanisms that are dependent on growth conditions and the genotype of the bacteria. *P. aeruginosa* strain PA14, when grown in low salt medium, kills worms over a period of 2-3 days by an infection-like process that correlates with the accumulation of bacteria in the worm gut (Tan et al., 1999). When PA14 is grown in a high salt medium that is akin to the CF lung, it kills worms within 4-24 hours by the production of low molecular weight toxin(s) (Mahajan-Miklos et al., 1999). Another strain of *P. aeruginosa*, PA01, kills rapidly by yet another mechanism. When PA01 is grown on brain-heart infusion agar, worms become paralyzed within 4 hours upon contact with the bacterial lawn (Darby et al., 1999). We showed that pathogenesis of *C. elegans* and mammals involves a shared set of *P. aeruginosa* virulence determinants (Mahajan-Miklos et al., 1999; Tan et al., 1999a,b). This suggests that the entire *P. aeruginosa* genome can be systematically, efficiently, and economically scanned for any gene that affects pathogenesis *in vivo* using *C. elegans*. We have performed screens for *P. aeruginosa* mutants that are attenuated in killing *C. elegans*. The screen identified known virulence factors such as the partners in a two-component regulator, *gacA* and *lemA*, and a regulator of quorum sensing, *lasR* that are also important virulence factors in other human pathogens. We will discuss other genes identified in the screen that encode novel virulence determinants and their role in pathogenesis.

We reasoned that because the host innate immune response is conserved between mammals and invertebrates, we can identify host factors that are involved in defense against pathogens by direct screening for *C. elegans* mutants that are either more resistant or more susceptible to pathogen attack. Analysis of *C. elegans*

mutants that are more susceptible to *P. aeruginosa* established that the nematode utilizes, among others, a conserved developmental pathway, a TGF β -like pathway, for defense against bacterial infections. The TGF β superfamily orchestrates vital roles in mammalian embryogenesis, organogenesis and immunity (Massague et al., 2000). In *C. elegans*, there are 2 well characterized TGF β pathways: the Daf and the Sma pathways (Patterson and Padgett, 2000). The Daf pathway regulates dauer larva formation in response to environmental cues. In contrast, the Sma pathway controls the nematode body length and ray formation in the male tail. Of the two pathways, components of the Sma pathway are more closely related to the mammalian TGF β pathway (Newfeld et al., 1999). We showed that the Sma, but not the Daf, pathway is also required to defend worms from pathogen. Mutations in the ligand *dbl-1*, or the Type I receptor *sma-6*, or any of the Smads (*sma-2,3,or 4*), rendered the worms more susceptible to *P. aeruginosa*-mediated killing, whereas mutation in neither *daf-7* nor *daf-5* has any effect. By phylogenetic comparison, the *C. elegans* SMA-2 and SMA-3 are more closely related to mammalian R-Smads, and SMA-4 to mammalian Co-Smad than any of the DAF proteins (Newfeld et al., 1999). In mice, targeted gene disruption of the Smad3 gene results in increased susceptibility to infection due to defects in mucosal immunity (Yang et al., 1999). Taken together, our results demonstrate that the function of the TGF β pathway in *C. elegans* immunity is also conserved in mammals. Therefore, the pathogenicity phenotypes can be used to identify and characterize novel components of the TGF β pathway that are essential for immunity.

We have also identified *C. elegans* genes that are transcriptionally regulated by the TGF β pathways in response to pathogen using a DNA microarray that contains about 96% of the 18,576 *C. elegans* genes (Jiang et al., 2001). We will discuss recent results obtained from functional analyses of a subset of these genes by double-stranded RNA interference (RNAi).

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S3.3

BACTERIAL FUNCTIONAL GENOMICS: STRATEGIES FOR IDENTIFYING DRUG TARGETS IN *PSEUDOMONAS AERUGINOSA*

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Pseudomonas aeruginosa infections of cystic fibrosis (CF) patients cause repeated episodes of airway obstruction and contribute to the progressive decrease in lung function that eventually kills the patient. Although current antibiotics and anti-inflammatory drugs can alleviate this to some extent, the infection can never be completely eradicated. Intensive research is therefore being devoted to developing novel therapeutics for the treatment of *P. aeruginosa* infections.

One major approach in this area is to identify *P. aeruginosa* genes that are essential for survival, and subsequently to produce agents that target those gene functions. Although this strategy has been pursued for a number of years, it was aided enormously by the recent completion of the *P. aeruginosa* genome sequence (Stover, et al., 2000) and the increasing application of computer and engineering procedures to biological research. These have enabled the development of genomic methods that are currently being employed to identify essential genes, including the use of conditional mutants, GAMBIT technologies, expression profiling, and comparative genomics.

Genome-wide conditional mutations were recently used to carry out a screen for essential *Vibrio cholerae* genes (Judson and Mekalanos, 2000). In that study, the *V. cholerae* genome was mutagenized with a transposon carrying an arabinose-inducible promoter (TnAraOut), and the resultant library was screened for strains with an arabinose-dependent growth phenotype. Three classes of genes were identified: genes essential for viability, genes that are not strictly essential but whose expression provides a growth advantage, and genes whose expression inhibits growth. The same approach is being employed in *P. aeruginosa*, where a TnOut library containing 900,000 clones has already been generated and is being screened for mutants that display an IPTG-dependent growth phenotype.

The *P. aeruginosa* TnOut library is also being used in a chip-based GAMBIT strategy. Originally developed in *Haemophilus influenzae*, GAMBIT combined saturating transposon mutagenesis with PCR footprinting to identify essential genes (Akerley, et al., 1998). In order to apply GAMBIT in *P. aeruginosa*, it has been adapted for use with DNA microarrays, and these modifications will also permit GAMBIT analysis of a wider variety of pathogenic organisms.

Expression profiling refers to methods in which RNA or cDNA is prepared from bacteria grown under different conditions and then hybridized to DNA microarrays to determine relative expression levels. These types of experiments have been carried out in numerous biological

systems, but few to date have examined gene expression in pathogenic bacteria. Operating on the hypothesis that genes that are more highly expressed during infection are likely to be required for survival in the host, this approach is being pursued in *P. aeruginosa* in an attempt to identify essential genes and to characterize the genetic regulatory networks that control virulence. Microarray analysis of host gene expression in response to *P. aeruginosa* infection is also being conducted.

Using comparative genomics to identify essential genes is based on the idea that gene homologues are likely to have similar roles even in different organisms. There is already a wealth of information regarding gene function in a variety of bacteria. This makes it possible to identify essential gene candidates in *P. aeruginosa* and to design appropriate experiments for determining their role in infection. Comparative genomics can also be used to examine genetic differences among environmental and clinical isolates, which could uncover genes that are critical for colonization and virulence. Such studies are facilitated by the ever-increasing number of completed bacterial genomes, and by the growing sophistication of the methods used to analyze those sequences.

In conclusion, genomic strategies are providing researchers with powerful new methods for understanding the genetic basis of how bacterial pathogens establish infections. Combined with proteomic and combinatorial chemistry approaches, this will aid the development of new therapies against *P. aeruginosa* infections in CF patients.

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S3.4

COMPARISONS OF THE GENOMES OF *P. AERUGINOSA* CF ISOLATES WITH THE PAO1 REFERENCE GENOME

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We have employed whole-genome-sample sequencing and DNA fingerprinting to compare the genomes of two CF isolates of *P. aeruginosa* and one environmental isolate with that of the fully sequenced reference strain PAO1. The CF strains belonged to a sequential series of isolates obtained from two CF patients over a period of years. We carried out the sample sequencing on the latest isolate available from each patient, while relying on DNA fingerprinting to determine whether or not the sampled isolate was typical of strains present earlier in the infection.

With a single exception, we found that all isolates from a particular CF patient had identical or near-identical DNA fingerprints. The sequence sampling involved approximately 10,000 sequencing traces from each strain, which provided approximately 0.5X coverage of each genome in high-quality base calls. At this sampling intensity, the probability of sampling a particular base pair in high-quality data is approximately 0.4. We classified discrepancies between sequence data on the sampled strains and the PAO1 reference sequence as due either to genetic variation or data errors based on quality statistics provided by the phred base-calling program (1).

Most of the sequence differences between the sampled strains and PAO1 are single-nucleotide polymorphisms (SNPs). The median frequency of SNPs in all pairwise comparisons between sampled strains and PAO1 is approximately 0.5%. However, at approximately 10-20 loci in each sampled strain, local sequence divergence relative to PAO1 is substantially higher than would be expected on the basis of random variation around this background level. In some cases, these hypervariable loci correspond to loci such as *fliD* at which major inter-strain divergence in sequence has already been described (2). However, several of the other loci represent novel examples of similar phenomena. A particularly dramatic locus, in which all the sampled strains are highly divergent from PAO1 is the *pvd* gene cluster, whose products are involved in pyoverdine synthesis (3).

With only a few exceptions, the distribution of sample-sequence data across the PAO1 gene conformed reasonably well to a random-sampling model. However, a few regions that are much longer than would be expected due to incomplete sampling lacked any alignable sequence data from one or more of the sampled strains. These gaps in the sample-sequence data arose in two different ways. In some instances the strains differ because

of substitutions of blocks of genomic sequence whose sequences are too divergent to allow inter-strain alignment. A well known example of this phenomenon is the cluster of genes that directs synthesis of disparate versions of the O-antigen (4). However, in other instances, individual sampled strains simply lack large segments of DNA found in the PAO1 genome and in the genomes of the other sampled strains. For example, one of the CF isolates lacks a segment of 100 kbp centered on coordinate 2.4 Mbp in the PAO1 reference sequence. The missing segment contains over 90 functionally diverse genes. PCR analysis indicated that the deletion is present in the entire set of isolates from this CF patient.

We did detect one major sequence variant that appears to have occurred following infection of one of the CF patients we studied. In the isolate for which the sample-sequence data were collected, we detected a transposon insertion in the cluster of genes that directs O-antigen synthesis. However, in this case, PCR analysis of the earliest available isolates from this patient indicated that the transposon was absent early in the infection even though DNA fingerprinting confirmed that no strain replacement had occurred. Hence, the transposon mutation is likely to have been selected during the infection. Loss of O-antigen synthesis is commonly observed in CF infections and earlier indirect evidence has been reported suggesting that this loss is at least sometimes due to mutation (4). The transposition event we detected is the first instance in which a mutation in this gene cluster that occurred in a CF patient has been detected.

This preliminary whole-genome survey of between-patient and within-patient genetic variation in *P. aeruginosa* infections of CF patients provide a starting point for relating genomic characteristics of bacteria isolated from CF patients to those of the well studied PAO1 strain. It builds on previous studies limited to a small number of genetic loci (5). We believe that further characterization of these genomic differences may contribute to the development of improved therapeutic regimens.

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S4.1

EPITHELIAL STEM CELLS: KEEPING AN EYE ON THE BULGE

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Epithelial stem cells are responsible for the homeostasis of all self-renewing tissues. Moreover they play a central role in wound repair and are the target cells for tumor initiation and certain strategies of gene therapy. An important primary concept in stem cell biology is the sequence of “stem cell → transit amplifying cell → terminally differentiated cell”. One of the most universally accepted criteria for epithelial stem cells is that they normally rarely cycle in vivo (slow-cycling) but have a high proliferative potential; that they can be activated by wounding to proliferate and to regenerate the tissue; that they are relatively small and primitive; and that they give rise to rapidly cycling transit amplifying (TA) cells, which have only a limited proliferative potential (for reviews see 1-3). The slow-cycling attribute is particularly important biologically because it conserves the cell’s proliferative capacity and minimizes DNA replication-related errors. The slow cycling nature of the stem cell means that these cells can be identified experimentally as the “label-retaining cells” (LRCs) (4 and references therein). Using this approach, it has been shown that, in murine epidermis, cells with stem cell characteristics are thought to be located at the center of an epidermal proliferative unit [5]. Within the hair follicle, the bulge region of the outer root sheath epithelium has been demonstrated to contain a subpopulation of cells with stem cell characteristics [4,6]. Finally, we have shown that the corneal epithelium contained no LRCs; such cells were found exclusively in the peripheral cornea in an area known as the limbus [7-9].

The limbal epithelium fulfills many of the criteria associated with epithelial stem cells (for a review see 2). Specifically, limbal basal layer contains a subpopulation of cells that rarely cycle [6,9]; they are biochemically primitive, lacking corneal epithelial differentiation-associated keratin K3 [7]; they have a high proliferative capacity both in vivo and in vitro [8,10,11]; they represent the predominant site of corneal tumor formation [12]; they are essential for the long-term maintenance of the corneal epithelium and can be used to reconstitute the entire corneal epithelium in patients with limbal

stem cell deficiencies (for reviews see 13, 14); they give rise to TA cells that undergo centripetal migration; they are located in a specialized “niche” characterized by a loose and well-vascularized stroma [8]; and they can rescue/reconstitute severely damaged or completely lost corneal epithelium upon transplantation [15]. Collectively, these data have led to the wide acceptance that corneal epithelial stem cells reside in the limbus.

Within the skin, the bulge zone of the hair follicle contains a population of cells that satisfy many of the existing criteria for keratinocyte stem cells [2]. The bulge is a part of the outer root sheath that is contiguous with the epidermis. It is the attachment site of the arrector pili muscle and marks the lowest end of the upper (permanent) portion of the follicle. Bulge cells are rarely cycling; have a higher proliferative capacity than the epidermis; have a primitive ultrastructure; are in contact with a specialized smooth muscle; are well-protected from the external environment; and are thought to be a major target of chemical carcinogens (see 2, 4 and references therein). Although it was customarily thought that the hair follicle and the epidermis were governed by separate populations of stem cells, it was puzzling that (1) very few stem cells (i.e., LRCs) were found in the interfollicular epidermis [4] and (2) interfollicular human epidermal cells had less in vitro proliferative potential than the upper follicular epithelial cells [16]. We showed recently that the bulge stem cells give rise not only to the lower follicle, but also to young transit amplifying cells that migrate into normal newborn mouse epidermis as well as wounded adult mouse skin. This provides the first evidence that the bulge represents a major repository of skin keratinocyte stem cells that may be bipotent as they can give rise to not only the hair follicle, but also the epidermis [4]. It is important to note that there are similarities between the limbal/corneal and follicular/epidermis stem cell systems. In the corneal/limbal system, the corneal epithelium is maintained via the limbal stem cells, which give rise to TA cells with a hierarchy of proliferative potential. These TA cells migrate centripetally towards the central corneal epithelium, and

in doing so progressively lose their proliferative potential. In the follicular/epidermis system, the epidermis is maintained in part by an upward flow of bulge-derived young TA cells into the epidermis.

The approaches that we have used to detect the stem cells and to carry out cell lineage analysis should be applicable also to other self-renewing systems such as the air-way epithelium. Information gained from such studies should greatly improve our understanding of the long-term maintenance of these tissues, the pathogenesis of diseases, and the regulation of wound repair.

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S4.2

EARLY COMMITMENT OF DISTINCT SUBSETS OF PROXIMAL AND DISTAL AIRWAY EPITHELIAL CELLS DURING FORMATION OF THE LUNG

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Respiratory epithelial cells lining the conducting alveolar regions of the lung are derived by differentiation and proliferation of precursor cells from the foregut endoderm. In order to discern lineage relationships among respiratory epithelial cells during lung morphogenesis, transgenic mice were generated in which respiratory epithelial progenitor cells were marked by doxycycline inducible expression of cre-recombinase, activating alkaline phosphatase expression by excision/ligation of loxP sites under control of the SP-C (surfactant protein C) promoter element. Continued exposure of the dam to doxycycline caused extensive labeling of all respiratory

epithelial cells in trachea, and nearly complete labeling of bronchial, bronchiolar, and alveolar cells in the newborn and adult mice. When labeled from E6.5-8.5, before formation of the lung bud, extensive labeling of all peripheral alveolar epithelial cells, including Type I and Type II epithelial cells, was observed while conducting airway cells were entirely unmarked, demonstrating the early and distinct commitment of proximal versus distal respiratory epithelial cells during formation of the lung. Surprisingly, recombination of conducting airway epithelial cells was observed during labeling from E10.5-12.5, identifying a latter period during which the SP-C

promoter defined a distinct subset of proximal progenitor cells and their derivatives. Proximal or peripheral respiratory epithelial cells are derived from distinct precursor cells committed early in the embryonic period, before formation of the lung buds. A distinct subset of respiratory epithelial cells, contributing to the respira-

tory epithelium of trachea and bronchi, are identified by SP-C dependent labeling later in lung morphogenesis. Cre-recombinase treatment of the animals with doxycycline in the postnatal period of development resulted in only rare recombination events, demonstrating the precise temporal nature of progenitor cells in the lung.

S4.3 UPPER AIRWAY STEM CELL NICHES AND CLONAL GROWTH OF PROGENITORS

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The airway epithelium is a crucial environmental interface that protects the lung from a battery of inhaled insults including toxic and infectious agents. However, phenotypic changes in the epithelium likely contribute to the descending spiral of physiologic impairment inherent to chronic lung diseases. For example, goblet cell hyperplasia and metaplasia is prominent in CF. Furthermore, it is widely assumed that the pseudostratified airway epithelium is an important target for CF gene therapy. Despite the pivotal role of the airway epithelium, the fundamental properties of which cells are the stem cells and predominant cell lineages remain poorly understood.

A conceptual framework for understanding cell lineage in airways. In renewing cell systems, the generally accepted paradigm is a three-compartment model consisting of stem, transiently-amplifying and terminally-differentiated cells (Fig. 1A). An important concept is that stem cells cycle slowly in order to sustain a lifetime of cell renewal and to protect against genetic damage during DNA replication. Most cell proliferation is accomplished by transiently-amplifying cells and stem cells are recruited only upon depletion of this intermediate compartment. Transient-amplifiers ultimately give rise to terminally-differentiated cells incapable of cell division. Tissue homeostasis is closely regulated by intricate control of proliferation, differentiation and cell

death. In well-understood organs such as the skin and gut there are specific spatial and temporal relationships between stem cells and their differentiated progeny (Fig. 1B). In several systems, stem cells occupy specific niches where they are maintained and regulated by complex cues between each other, their descendants, extracellular matrix and underlying mesenchymal cells (3).

Implications for gene therapy. Gene therapy for CF using integrative vectors holds the promise of long term cure. However, a lasting effect requires transfection of progenitor cells and expression of CFTR in their differentiated daughters. Application of a "stem cell niche" model, to the pseudostratified airway epithelium predicts a relatively rare population of potentially inaccessible stem cells (Fig. 2). However, a unique subpopulation of airway cells with very high growth capacities has not been identified. In fact, the epithelium is strikingly remodeled in disease, which is regarded as plasticity (4). Several cell types proliferate during remodeling and daughter cells may follow different differentiation pathways than their parents. This great plasticity suggests an alternative, "unlimited plasticity" model where many non-terminal cells are capable of nearly equivalent levels of growth and differentiation under stress. Thus, there is no specialized stem cell compartment. This model implies that broad targeting of the relatively large pool of highly plastic progenitors is preferable, with persistence

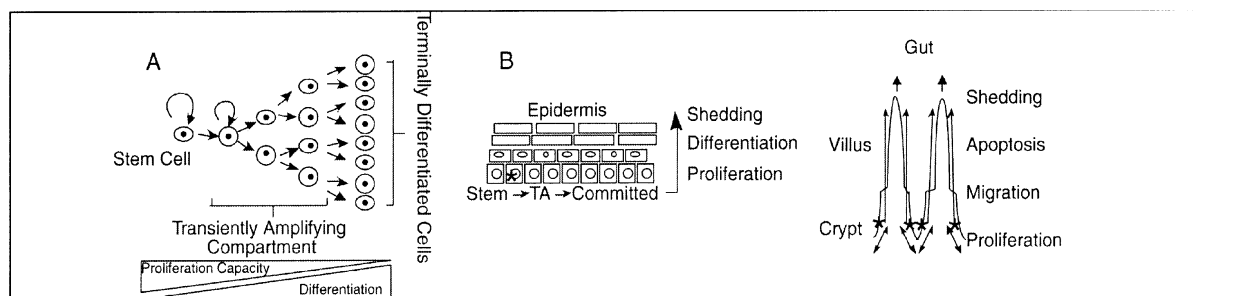


Fig. 1. A) Three compartment model of cell lineages. B) Relationships of epithelial stem cells (*) to their progeny in well-studied organs. Adapted from (1) and (2).

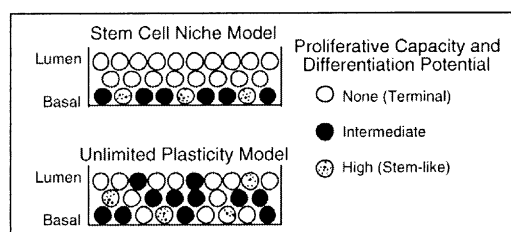


Fig. 2. Two theoretical models of cell lineage in the pseudostratified airway epithelium. The “stem cell niche” model predicts a small population of stem cells in a distinct morphologic compartment. In the “unlimited plasticity” model, many cells with ample progenitorial capacity are scattered throughout the epithelium. Many variations between these extremes are possible.

being determined by the probability of transfected cells being called upon to proliferate, differentiate and die.

What conclusions regarding the relative applicability of these two contrasting models can be drawn from the current data? While controversial, the preponderance of the evidence is that cells within both the basal and secretory cell compartments of the pseudostratified airway epithelium divide and are multipotent progenitors. However, available data does not conclusively distinguish between the theoretical models presented above. While there is substantial proliferative reserve in many different airway epithelial cell types, there may also be stem cells, which are only recruited under extreme conditions of epithelial damage.

Evidence for stem cell niches. In studies aiming to isolate specific subpopulations of murine tracheal epithelial cells, we discovered a distinct population of cells displaying high levels of keratin expression in murine tracheal submucosal gland ducts. We tested the hypothesis that bromodeoxyuridine (BrdU) label-retaining cells (LRCs), thought to represent stem cells, were present in this compartment. Mice received weekly epithelial damage by intra-tracheal detergent or SO₂ inhalation for four weeks and received IP injections of BrdU every 48 hours during the injury and repair period. Three and six days post injury, BrdU-positive epithelial cells were noted along the entire tracheal length in both basal and luminal cell positions. At later time points (20 and 95 days) LRCs were localized to gland ducts in the upper trachea and to systematically arrayed foci in the lower trachea, typically near the cartilage-intercartilage junction. LRCs were not pulmonary neuroendocrine cells. Heterotopic tracheal grafts following surface epithelial removal demonstrated reconstitution of a surface-like epithelium from gland remnants (5). These results suggest that airway epithelial stem cells are localized to specific niches.

Clonal growth of isolated sub-populations of airway epithelial cells. One approach for studying cell lineages has been to dissociate the epithelium, separate the cells into

subpopulations and examine their growth and differentiation. Previous studies clearly indicated that both basal and columnar cell types could serve as multipotent progenitors (6). Retrovirally tagged human bronchial cells inoculated into tracheal grafts displayed a restricted pattern of colony types suggesting that basal cells and intermediate cells were the predominant progenitors (7). However, clonal growth of isolated sub-populations has not been studied. We isolated basal and columnar cells from protease XIV-dissociated Rosa26 mouse ((gal in all cells) tracheas by flow cytometry using either GSIA3B lectin or transgenic mice expressing GFP driven by the basal-cell-specific keratin 5 promoter (K5GFP). Isolated cells were mixed with non-Rosa cells at varying dilutions and were seeded into denuded tracheas for heterotopic transplantation *in vivo* or into an air-liquid interface epithelial-fibroblast co-culture system *in vitro*. After a mucociliary epithelium was re-established, clonal growth was elucidated by (gal staining. The total number of clones, as well as the number of large clones, per tracheal graft *in vivo* was identical between lectin-sorted basal and columnar cells. For both lectin- and K5GFP-sorted populations *in vitro*, clonal efficiency and the number of large clones derived from the basal cells was approximately double that of columnar cells. These results indicate that cells with an adequate progenitorial capacity for clonal expansion *in vivo* and *in vitro* are not restricted to distinct morphologic compartments of the epithelium.

Summary. Understanding cell lineages in the airway epithelium is generally important, and vital for gene therapy, but stem cells have not been identified. *In vivo* studies of injured mouse tracheas suggest the presence of stem cell niches. *In vitro*, isolated mouse tracheal epithelial cell populations highly enriched with basal cells have approximately double the growth potential of non-basal cells while *in vivo*, the two cell types appear to grow equivalently. However, the utilized assays of growth potential may not be “acid tests” for stem cells. Furthermore, the upper and lower trachea may represent related, but different “zones” with distinct cell populations and cell lineage patterns. Thus, further study is needed to better define and localize airway epithelial stem cells.

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S4.4 STEM CELLS AND STEM CELL MICROENVIRONMENTS OF THE DISTAL CONDUCTING AIRWAY

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Airway remodeling, of which changes to epithelial cells are becoming increasingly recognized as a critical component, is common to many chronic airway diseases including cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) and asthma. Many distinct factors can influence airway remodeling including the cytokine milieu resulting from infection and/or inflammation, and cellular injury involving either terminally differentiated or progenitor cell populations. Cell types contributing to epithelial renewal can be broadly categorized as either stem cells, a slow-cycling, relatively undifferentiated population of regenerative cells with pluripotent differentiation potential and unlimited proliferative capacity, or transit amplifying (TA) cells, which are generally more abundant lineage committed progenitor cells with finite proliferative capacity. Even though stem cells for the pulmonary epithelium have been postulated to exist, their location and molecular phenotype has remained elusive (Mason et al., 1997). Identification of lung stem cells, their molecular phenotype and permissive microenvironments for their maintenance, will greatly facilitate our understanding of pathological mechanisms in airway diseases and may provide new insights into therapeutic approaches for amelioration of disease.

Mechanisms of tissue maintenance and renewal differ between organs. In contrast to tissues such as the cornea, skin and intestinal epithelium, which harbor highly mitotic cell populations in the steady-state, organs such as the lung and liver have a low cellular mitotic index in the steady-state which only increases dramatically following injury. Nonciliated airway epithelial (Clara) cells represent an abundant proliferative population of the human bronchiolar epithelium and are the principal regenerative cell type of the rodent conducting airway (Khor et al., 1996; Evans et al., 1978). Clara cells have many characteristics of TA cells in that they fulfill several differentiated functions in the steady state and enter the cell cycle following injury. Differentiated functions of Clara cells, such as phase I pollutant metabolism, render them sensitive to injury by lipophilic pollutants. Conditions of progenitor cell depletion, such as that achieved through either mechanical or chemical injury, have been used for the activation of latent stem cell populations allowing their localization and characterization.

We have used naphthalene exposure, a pollutant metabolized to cytotoxic epoxides through cytochrome P450-catalyzed reactions, to effect Clara cell depletion in mice with the goal of activating latent stem cells of the

conducting airway epithelium. Parenteral exposure of mice to 250 mg/kg naphthalene results in Clara cell necrosis and a dramatic decrease in the abundance of the Clara cell-specific gene product Clara cell secretory protein (CCSP) mRNA within total lung RNA. Airway repair was characterized by restoration of depleted CCSP-expressing (CE) cells. Naphthalene-resistant regenerative foci localized to discrete sites within the bronchiolar epithelium that colocalized with calcitonin gene-related peptide (CGRP)-immunoreactive cells organized into neuroepithelial bodies (NEB's) (Stripp et al., 1995; Reynolds et al., 2000). In contrast, renewal of the terminal bronchiolar epithelium initiated through proliferation of cells at the broncho-alveolar duct junction (BADJ). Slow cycling cells were identified through measurement of the rate at which tritiated thymidine was diluted from the DNA of proliferative cells labeled early in the recovery phase following naphthalene exposure. Two distinct cell types showed label retention within the NEB microenvironment, pulmonary neuroendocrine cells and a subpopulation of CCSP-expressing cells (Hong et al., 2001). Label retaining cells of the terminal bronchiolar microenvironment localized to the BADJ and expressed CCSP. Based upon these findings, two candidate stem cell types existed within the NEB microenvironment and a pollutant-resistant CE population represented the putative stem cell of the terminal bronchiolar epithelium. Contributions of CE cells versus other proliferative cell types to renewal of the bronchiolar epithelium were determined through generation of transgenic mice in which the *Herpes simplex* virus thymidine kinase gene was expressed specifically within CE cells under the control of the mouse CCSP promoter (CCSP-HSVtk). Exposure of CCSP-HSVtk transgenic mice to ganciclovir (GCV) allowed temporally controlled conditional ablation of CE cells. Acute exposure of adult CCSP-HSVtk transgenic mice to GCV resulted in rapid depletion of CE cells throughout the bronchiolar, bronchial and tracheal epithelium. Recovering mice exhibited proliferation and hyperplasia of pulmonary neuroendocrine cells. However, proliferative cell types other than pulmonary neuroendocrine cells were not observed within the bronchiolar epithelium. Moreover, epithelia depleted of CE cells failed to an epithelium of appropriate composition.

We conclude that cells with a relatively undifferentiated (pollutant-resistant), slow-cycling phenotype exist within conducting airways. These regenerative cells are maintained within multiple seemingly disparate microenvironments throughout the conducting airway, including

BADJ and NEB-microenvironments of the bronchiolar epithelium, and are activated following TA cell depletion. Putative stem cells maintained within BADJ and NEB microenvironments either express CCSP or require CCSP-expressing cells to support their participation in epithelial renewal. Ongoing studies are aimed at further defining the molecular phenotype of stem cells within the bronchiolar epithelium and mechanisms contributing to their maintenance in the developing and adult lung.

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S4.5

INTRINSIC FACTORS CONTROLLING SUBMUCOSAL GLAND STEM CELL PHENOTYPES

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Submucosal glands in the airway, which express high levels of CFTR protein, represent a potentially important site of pathology in cystic fibrosis (CF) lung disease. Gene targeting to submucosal glands in the adult will likely be extremely difficult should therapeutic intervention at these inaccessible sites be required to treat CF lung disease. To this end, we are attempting to more clearly define submucosal gland stem cell phenotypes in an effort to establish rational strategies to target these cells by in utero gene therapy. Functional retroviral marking studies have established that submucosal gland stem cells do exist in the human surface airway epithelium with pluripotent capacity for both surface airway and submucosal gland epithelial differentiation (Engelhardt et al., 1995). Additionally, we have defined the requirements of a submucosal gland specific stem cell factor called LEF-1 which is specifically expressed in gland stem cells at the time of commitment to form submucosal glands (Duan et al., 1998). LEF-1 expression is functionally required for airway submucosal gland formation in mice and ferret trachea (Duan et al., 1999).

In an effort to more clearly understand the regulatory mechanisms that control LEF-1 induction during gland development, we have cloned the LEF-1 promoter and have begun to characterize its regulatory properties in vitro and in transgenic mice. One family of developmental factors that has been a focus of recent studies includes

Wnts. Members of the Wnt family of secreted molecules have been established as key factors in determining cell fate and morphogenic signaling. During development, signaling molecules such as wnt are expressed in both spatially and temporally regulated manners and control a number of complex interactions important in development. It has long been recognized that Wnt induces morphogenic signaling through the Tcf/LEF-1/ β -catenin cascade. Predominantly, this induction is thought to be controlled through regulation of free intracellular levels of β -catenin, a co-factor for Tcf/LEF-1 transcription factors. Furthermore, numerous Tcf/LEF-1 binding sites exist in the LEF-1 promoter and are likely candidates for its coordinated expression during gland development. Using in vitro expression analyses with defined LEF-1 promoter reporter constructs, we have demonstrated that Wnt-3A can induce LEF-1 promoter transcription and that this induction is dependent on a short 110bp region of the promoter termed the Wnt responsive element (WRE). Activation of the LEF-1 promoter by Wnt-3A was dependent on an increase in free intracellular β -catenin mediated through the GSK-3 β pathway. Interestingly, induction through this pathway controlled LEF-1 promoter activity by relieving repressor functions confined to the WRE. Furthermore, Wnt-3A induction was associated with alternative transcriptional initiation of two of the four transcription start sites in the promoter.

Studies attempt to define the *in vivo* promoter elements required for LEF-1 induction in developmental morphogenic responses have utilized transgenic mice expressing LEF-1 promoter/LacZ reporters. Although visualization LEF-1 promoter activity in submucosal gland stem cells was difficult due to their infrequent abundance in the mouse, evidence of tissue specific expression was noted in several functionally similar models of epithelial invasion and morphogenesis such as whiskers and hair follicles. Like submucosal glands, both whisker and hair follicle development are also dependent on functional LEF-1 as indicated in transgenic knock-out mice.

In summary, our findings suggest that Wnt-3A mediated enhancement of LEF-1 promoter activity may occur through a mechanism of derepression involving β -catenin and alternative transcriptional initiation. These results provide the first definitive evidence for direct regulation of the LEF-1 promoter by Wnt and provide a

clearer mechanistic foundation for Wnt/LEF-1 signaling in the regulation of developmental processes such as submucosal gland morphogenesis.

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S5.1

THE IMPACT OF NUTRITION ON PULMONARY FUNCTION AND SURVIVAL IN CF PATIENTS

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There are hundreds of descriptive studies showing a correlation of nutritional status with pulmonary function, and dozens more suggesting that improved survival is associated with changes in dietary management. The strength and direction of the association of declining FEV₁ with mortality are overwhelming (1,2). How nutritional status impacts on this causal pathway is not so clear-cut. The evidence that nutrition can affect - not just reflect - the course of lung disease and ultimate survival is compelling, although based primarily on cohort and descriptive studies. Representative studies have been selected to illustrate the changing approach to nutritional management and its impact on disease course in CF.

The earliest evidence that it was safe to abandon the restricted fat diet that had been prescribed for several decades came from Crozier's report of a "high fat" diet (3) as part of an aggressive treatment program for CF. A comparison of the clinic populations of Boston and Toronto (4) suggested that improved survival and growth in the Toronto patients was related to the change in diet, since other aspects of treatment were similar. The unrestricted high energy diet was gradually accepted and evaluated (5) and coincided with steadily improving survival in the US (2) and elsewhere. A recent population study (6) showed that the gap in height and weight distributions between the US and Canada was disappearing, but there were still alarming deficits in many age groups in both populations. Another study

(7) demonstrated what appeared to be worsening average nutritional status in a clinic population over a decade of improved nutrition, but also pointed to the increased survival of more severely affected patients as a complicating factor.

Survival regression studies (1,2) have shown that nutritional indices were highly predictive of survival in single variable models. But when FEV₁ was added to models, the magnitude and significance of the nutritional variable was greatly reduced. This does not necessarily mean that nutritional status is not an important predictor, only that it is inextricably confounded with the lung function effect. It may be that the old pattern of CF disease was that poor weight was an intermediate effect in the causal pathway of declining lung function to early mortality. But the result of successful nutritional interventions may produce a new causal pathway in which improved nutrition causes stabilized or improved pulmonary function resulting in delayed mortality. FEV₁ would still be the overwhelming predictor in regression models but the confounding dynamics would be different. Survival models that incorporate individual time trends of FEV₁ and height and weight indices are being developed and should help to sort out the relative contribution of these predictors of mortality.

New analytical approaches allow the simultaneous analysis of longitudinal changes in growth and pulmonary function in large populations (8) but interpretation

of causality in the interrelationship of nutritional and pulmonary decline is still problematic. Nutritional intervention studies of caloric supplements (9) and overnight enteral feeding (10) have clearly shown successful gains in weight and height, sometimes accompanied by signs of retarded progress of lung disease. But such studies are largely uncontrolled because placebo treatments are either impractical or considered unethical. Historic or pre/post treatment comparisons can be impressive but are subject to sources of bias relating to temporal trends and well-intentioned efforts to maximize the effects of a new treatment. The historical improvement in survival worldwide as more aggressive attempts at increasing caloric intake were generally adopted is impressive. Quantifying the impact on disease progression of more specific nutritional interventions will require more classical controlled clinical trials. At the same time, longitudinal regression analysis provides a tool whereby multiple variables can be assessed as predictors of outcome following a particular treatment protocol. Depending on the objectivity and consistency of the protocol usage, these statistical models will help to refine the application and timing of nutritional options that are believed or proved to be beneficial.

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S5.2

CURRENT NUTRITIONAL STATUS OF PATIENTS IN THE US

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Ten years ago the first nutrition consensus conference was sponsored by the Cystic Fibrosis Foundation, and shortly thereafter a set of guidelines was published.¹ There has been good progress in nutritional status and survival of CF patients since then, but there remains significant variability in compliance with the guidelines and CF patients still lag far behind the healthy population in attained growth. As the CF patient population ages, cystic fibrosis related diabetes mellitus is becoming an important challenge for patients and clinicians alike, with a new set of guidelines for screening and management.²

In 1989, the median age of CF patients in the US CFF Registry was 12 years and 31% were adults. In 1999, the median age is 14.5 and 38% are adults. The proportion at less than the 5th NCHS weight percentile has decreased from 30% to 21%. In the 1999 registry, 53% of patients are male, 96% are Caucasian, 52% are homozygous

(F508, 19% present with meconium ileus, and 90% take pancreatic enzymes.³

There is some controversy over whether CF patients can attain the growth potential of the normal population,⁴ but many studies have shown improved growth when the causes of chronic under nutrition are treated appropriately, and this has been associated with increased overall survival.⁵ In 1988, Mary Corey et. al. reported a comparison of Boston and Toronto patients which showed the Toronto patients to be considerably less underweight and with a longer median survival.⁶ Comparison of US and Canadian 1997 Registry data shows that US patients have made up much of the nutritional advantage seen in Canadian patients in the 1988 Corey study, perhaps in part as a result of the dissemination of the high-calorie, high-protein, unrestricted fat diet advocated in Toronto and in the CFF Clinical Practice Guidelines.⁷

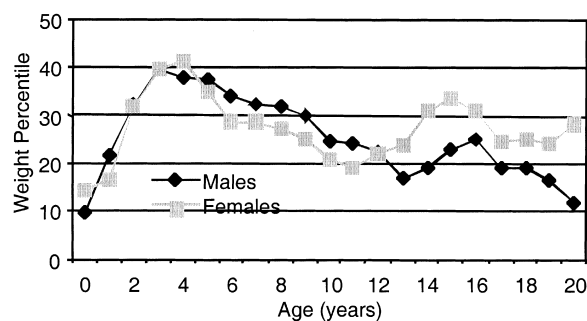


Figure 1. Median Centers for Disease Control Weight Percentile versus Age, by Sex

Currently, the US CF population median weight never rises past the 40th NCHS percentile, and steadily declines from the age of 6 to around the 20th percentile at age 20.³ The introduction of the new Centers for Disease Control growth charts has almost no impact on this profile (Figure 1). Body mass index percentiles also steadily decline from 50th percentile at age 5 to the 20th percentile at age 20. It is important to note that both BMI and Ideal Body Weight, traditional measures in the nutritional assessment of CF patients, are both weight-for-height measures, so when a patient is at the 15th percentile for weight and the 10th percentile for height, growth failure is hidden.

Analysis of 1999 CFF registry data reveals considerable variability in treatment of low body weight between CF Care Centers. The national rate of supplemental feeding (gastrostomy, nasogastric tube, parenteral, or jejunostomy) in children at less than the 5th NCHS weight percentile is 26.5% but the range is 0 to 71%, and only 14 centers reach 50%.

As CF patients live longer glucose intolerance and CF related diabetes (CFRD) are becoming common complications; 12% of patients over 13 years of age have chronic insulin dependent diabetes.² CFRD is associated with significantly increased rates of underweight, more severe pulmonary disease, and reduced survival.⁸ The 1998 Consensus Conference on CF-related Diabetes Mellitus² recommends screening all patients 14

years and older, but this remains an area of wide variability between CF Care Centers. Some centers report screening 100% of non-diabetic CF patients ≥ 14 years, and some report none screened, producing an apparent range of CFRD rates between centers in the screened population of 0 to 36%.

The nutritional status of US CF patients has improved since the publication of the first Consensus Conferences on nutritional assessment and management in 1990 and diagnosis and management of CFRD in 1999. The variation that persists in the management of CF patients produces a great opportunity to further reduce undernourishment in CF patients by closing the gaps between knowledge and current practice.

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