**Suppression of Premature Stop Codons for the Treatment of a Subset of Patients with Genetic Disorders**

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The concept of personalized medicine has been extended to recognize the fact that variations in the genetic makeup of individuals can greatly alter their responsiveness to drug treatments for a variety of disorders. An important corollary to this concept is that patients may have one of a number of different genetic diseases due to the same type of mutation in the disease-causing gene. This creates the possibility of a very different paradigm within personalized medicine, in which a drug can be designed to treat a specific genetic mutation, rather than a specific disease. The first example of this paradigm is drug-based suppression of a premature stop codon. There are a large number of individuals collectively afflicted by genetic disorders resulting from premature stop codons. Development of a drug-based therapy that can suppress this class of mutations could allow treatment of subsets of patients across a large number of genetic disorders, and thus address a substantial unmet medical need.

Within the patient populations of genetic disorders that arise as a consequence of mutations that result in the loss of a specific protein, premature stop codons (so-called nonsense mutations) are often the underlying defect. These mutations, creating a UAA, UAG, or UGA codon in the coding region of the mRNA, result in premature translational termination. This causes production of a truncated protein that is non-functional and/or rapidly degraded. Furthermore, premature stop codons lead to mRNA destabilization through the process of nonsense-mediated decay (NMD) of mRNA. Premature stop codons typically account for 5 to 20% of the individual cases of genetic disorders, including several cancers, and their incidence is considerably higher (up to 70%) in populations with founder effects.

Promoting read-through of premature stop codons with pharmacological agents was first investigated using aminoglycoside antibiotics. These drugs normally function by inhibiting bacterial protein synthesis at concentrations that do not alter protein synthesis in eukaryotic cells. However, it was first noted in yeast in the late 1970’s, that higher concentrations of aminoglycosides could lead to suppression of premature stop codons in yeast. These observations were extended to mammalian cells. This work first raised the possibility of the premature stop codon suppression as a possible treatment for human diseases, such as forms of thalassemia. These observations were later extended to human cells.

The first proof of principle experiments involving premature stop codon suppression in an animal model of a human disease utilized the mdx mouse. This mouse is a model of the human disease, Duchenne muscular dystrophy, and importantly not only displays premature translational termination resulting in the
total loss of the protein known as dystrophin, but also shows significant nonsense mediated decay of the affected mRNA.\textsuperscript{13} Thus this animal model faithfully reproduces both aspects of the disease-causing mutation that will be encountered in patients with disorders arising from premature stop codons.

The ability to suppress the effects of a disease-causing mutation and to generate therapeutic levels of missing protein with administration of the aminoglycoside, gentamicin generated considerable interest in moving toward clinical trials.\textsuperscript{14} The gentamicin study in the mdx mouse\textsuperscript{12} also provided insight into the types of diseases that might benefit from suppression of premature stop codons, stemming from the observation that production of the missing protein was at lower than normal rates and amounts. This was likely due to a combination of the suppression being inefficient and the mRNA levels being reduced (by NMD). This implied that there are at least two distinct scenarios in which premature stop codon therapies may be maximally effective: (1) Diseases in which a much lower than normal levels of the missing protein will be therapeutic, and (2) diseases in which the therapeutic protein has a very long half life and thus can accumulate to significant levels via premature stop codon suppression. Cystic fibrosis\textsuperscript{15} and hemophilia\textsuperscript{16} are examples of the first disease scenario, while Duchenne muscular dystrophy is an example of the second.\textsuperscript{12}

Subsequent to the initial mdx mouse studies,\textsuperscript{12} there were numerous studies performed in cultured cells and in mice aimed at demonstrating the feasibility of suppressing various human disease-causing premature stop mutations with aminoglycosides.\textsuperscript{16-28} Importantly, a number of clinical trials also followed, using gentamicin in patients with diseases resulting from premature stop codons.\textsuperscript{16,29-33} For example, topical application of gentamicin to the nasal mucosa of nonsense mutation-mediated cystic fibrosis (CF) patients for 14 days resulted in local CFTR protein production of the missing protein (CFTR) and functional improvements (chloride channel activity) resulting from the correct localization of the protein.\textsuperscript{29-31} Similarly, intravenous gentamicin treatment in patients with stop codons in Duchenne muscular dystrophy\textsuperscript{32} and hemophilia\textsuperscript{16} promoted production of the missing protein.

The gentamicin proof-of-concept clinical experiments demonstrated that small molecules can promote read-through of premature stop codons and thus have the potential to suppress the disease-causing mutation in patients whose disease is caused by a premature stop mutation. However, the lack of oral bioavailability and the potential for serious renal and otic toxicities limit the clinical utility aminoglycoside therapy in a chronic disease. Indeed, positive results were not uniformly obtained in trials with gentamicin,\textsuperscript{33} perhaps due to either under-dosing of the drug because of concerns with toxicity, or due to the variable heterogeneity of the gentamicin formulations.\textsuperscript{34} Thus there was a clear need for a new class of drug, preferably not an antibiotic, that would be orally bioavailable and low in toxicity, and yet suppress premature stop codons with high enough efficiency to produce therapeutic levels of disease-associated proteins.

This need may have been met with the development of PTC 124.\textsuperscript{35} PTC124 is a small molecule (<300 Da) that can be given orally and has shown low toxicity in animals and humans.\textsuperscript{35,36} The drug was developed using high throughput screens with premature stop codons inserted into reporter genes in cultured cells, and then refined with medicinal chemistry efforts that utilized the mdx mouse as a human disease model. In experiments that paralleled the gentamicin studies in the mdx mouse, PTC124 was shown to produce dystrophin protein at levels sufficient to correct the major features of the disease phenotype.\textsuperscript{35} Furthermore, experiments revealed that PTC selectivity suppresses premature stop codons, and not authentic stop codons, which likely is critical for the lack of toxicity. Based on this work, PTC124 has entered human trials in both Duchenne muscular dystrophy and Cystic Fi-
brosis. Interim Phase 2 results have been made available (http://www.ptcbio.com/3.1.1_genetic_disorders.aspx), and document production of therapeutic protein, as well as indications of efficacy, in a significant number of patients.

In summary, PTC124 may provide the first effective drug treatment for large number of patients afflicted with one of thousands of genetic diseases that can be caused by premature stop codons. As such it may allow us to forge a new paradigm in personalized medicine, in which patients are treated for a specific type of genetic mutation, rather than a specific disease.

References


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Molecular Genetics of Human Developmental Brain Disorders of the Arabian Gulf Region

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Abstract

Recent work has led to the genetic understanding of an increasingly large number of neurological disorders that affect the nervous system of children, resulting in symptoms such as mental retardation, autistic symptoms, cerebral palsy, or seizures. These conditions are extremely heterogeneous genetically, meaning that large numbers of genes appear to be essential for normal nervous system function, and that mutations in different genes can sometimes cause very similar symptoms. The larger family size, recent population growth, and unique ancestry of populations in the Gulf region provide special opportunities for understanding the causes of childhood neurological disease both here and throughout the world. Here I review some recent developments, and discuss their impact on therapy.

Recent work has identified the genetic causes of many developmental brain disorders, which is a term that encompasses a wide range of childhood neurological illness including mental retardation, seizures, cerebral palsy, autism, and other behavioral disorders. There is an increased appreciation that these disorders are extremely heterogeneous in their cause. For instance, there are known environmental causes, such as neonatal birth injury, that can result in cerebral palsy, seizures, or in some cases autistic symptoms. On the other hand, a wide range of studies indicates that the majority of these conditions have a genetic basis, though the identification of the precise genes that are responsible is far from complete.

Recent work on the genetic causes of developmental brain disorders has shown roles for genes with many different patterns of inheritance, including dominant, X-linked, and recessive. Moreover, the responsible genes show some regional specificity, depending on factors as genetic “founder” mutations (i.e., mutations that are more prevalent in one population than another because these mutations were present in the small, “founder” population from which a larger but geographically isolated population derived, e.g., Finland or Iceland). Other causes of regional differences in the genes associated with childhood brain disorders include differences in patterns of consanguinity, since marriage between distant relatives increases the prevalence of recessive mutations as a cause of disease.1

The dry desert conditions and patterns of tribal heritages of many nations in the Gulf region create many advantageous opportunities for mapping and cloning genes for recessive disorders of the brain, and Gulf populations have led the way in the identification of many recessive disease genes in the last five years. “Founder” mutations are characteristic of many geographically isolated populations
around the world, but are particularly commonly observed in the Gulf region; because of the recent, rapid increase in the size of the population in the last century. I review some of the conditions whose genetic cause has become clear in the last few years. Review of these disorders shows how the study of the genetic causes of childhood brain disease in Gulf populations has been very important for the diagnosis and treatment of children in the Gulf. However, since genes that were first identified by the study of Gulf region families have been shown to affect children around the world, further collaboration between physicians and scientists worldwide has the ability to greatly enhance our understanding of these conditions.

Microcephaly

Primary microcephaly. A common form of neurological disability in Gulf countries is primary microcephaly, which is defined as small head size <3 standard deviations below the mean for age and gender. Typically, primary microcephaly is defined by a relatively benign clinical picture, in which there is mild to moderate mental retardation, but often little delay in motor milestones. Seizures are not present as a rule, but occasional exceptions are seen. Although microcephaly is a physical sign that can reflect a large diversity of genetic and non genetic conditions, primary microcephaly is a fairly uniform diagnosis. On MRI, these patients usually show evidence for reduced size of the cerebral cortex, and because of that, the folding pattern of the cortex, consisting of gyri (folds) and sulci (fissures), shows moderate simplification, but other aspects of the shape of the cortex are relatively maintained. Over the past 5 years, many single gene mutations have been identified that cause primary microcephaly, in most cases because of the analysis of pedigrees from Pakistan or the Gulf region. MCPH 1-5 were originally mapped in Pakistani families, and ASPM mutations were found to be the most common cause of MCPH. ASPM mutations have been seen in families from Saudi Arabia and other Gulf countries. Microcephalin mutations cause MCPH1, whereas mutations in CDK5RAP2 and CENPJ cause MCPH3 and MCPH6, respectively. Clinical DNA-based diagnosis of these conditions has not been widely offered yet, so that the prevalence of these conditions in Western European populations and the USA is not known; however, since microcephaly is common worldwide, and since these genes are the most commonly affected genes, they are likely to be common, world-wide causes of this condition.

Cohen syndrome. One of the most common syndromic forms of microcephaly (meaning microcephaly along with other signs that allow the recognition of a broader syndrome) is known as Cohen syndrome. Whereas this condition has been observed particularly frequently in the Finnish population, due the occurrence of a founder mutation (Kolehmainen et al., 2003; Kolehmainen et al., 1997), our own work showed that this condition is also seen in the GCC, often with a somewhat atypical presentation (Mochida et al., 2004). Cohen syndrome presents with microcephaly and mental retardation, global developmental delay, severe myopia, neutropenia, retinal changes (consisting of pigmentary retinopathy and/or myopia), and a characteristic facial dysmorphism. The face shows downslanting palpebral fissures, “wave-shaped” eyelids, and a prominent nose, with a short and upturned philtrum. The genetic locus for Cohen syndrome was first mapped in Finnish pedigrees. Once the gene was identified as COH1, we collaborated with physicians and families from Saudi Arabia and Oman, and identified COH1 mutations similar to those reported in European families, but with a facial appearance that is somewhat different, explaining the lack of initial recognition of Cohen syndrome. With the application of DNA-based diagnostic testing, Cohen syndrome is now recognized as another recessive form of microcephaly with a
particularly high prevalence in the GCC countries.

**Microcephaly/periventricular heterotopia.** We recently described a form of microcephaly that is associated with more severe radiographic abnormalities on MRI scan, and a much more severe clinical course. In addition to severe microcephaly, these patients have seizures that begin at an early age and become intractable. These patients usually die young. On MRI scan there are nodules of cells with the MRI signal characteristics of neurons lining the lateral ventricle, referred to as periventricular heterotopia. This syndrome has been mapped and cloned in our laboratory, and reflects mutations in the ARFGEF2 gene, which encodes a GTPase exchange factor (GEF) for the small G protein, ARF (ADP ribosylation factor) that regulates vesicle trafficking and protein sorting. The second most common genetic cause of lissencephaly was discovered by our lab, and represents mutations in a gene called doublecortin (DCX), which is an X-linked gene that causes a milder phenotype in females than in males.

However, in a series of patients from Jordan (al-Qudah, 1998), consanguinity was noted to be very common in patients with lissencephaly, suggesting that additional, recessive genes may be causative in this population, though less common in the European or American population. Our lab has made considerable progress in describing these recessive genes that are important in the Gulf area.

**Lissencephaly with cerebellar hypoplasia.** One recessively inherited form of lissencephaly is associated with a severe malformation of the brainstem and cerebellum. First described in a British family in which the parents were half-first cousins, this condition was subsequently described by Al Shawan in Saudi Arabia in a consanguineous family. We collaborated with these two groups to map this disorder, and identify causative mutations in the Reelin gene (RELN), which encodes a large extracellular protein that guides migrating neurons to the cerebral cortex.

**Walker- Warburg syndrome/ cobblestone lissencephaly.** Other important causes of lissencephaly are associated with disruption of eye and muscle development as well. There are several such syndromes, all recessively inherited. Fukuyama congenital muscular dystrophy (FCMD) is a syndrome first described in Japan, and largely limited to this country, because of the existence of a special founder mutation in the responsible gene, known as Fukutin. A second disorder, known as Muscle-Eye-Brain disease, and first described by Santovouri, shows many similarities to FCMD, but is due to mutations in a distinct gene, POMGnTI. Walker Warbug syndrome (WWS) is more severe than either FCMD or MEB, since it typically...
shows neonatal onset of seizures and extreme hypotonia and early death. Our analysis of Gulf region families with WWS allowed the identification of POMT1 and POMT2 mutations in WWS, and an assessment of how frequent POMT1 mutations are as a cause of WWS. Additional genes for this syndrome remain to be identified.

**Bilateral frontoparietal polymicrogyria (BFPP).**
A disorder that is commonly diagnosed as lissencephaly, but that is actually a distinct syndrome pathologically and radiographically, is known as polymicrogyria, since the gyri are actually overly numerous and reduced in size. We mapped the first form of polymicrogyria that involves predominantly the frontal and parietal lobes, known as BFPP, in two Palestinian families, and identified the genetic cause as mutations in the GPR56 gene. The availability of genetic testing for this disorder has allowed us to identify many children with GPR56 mutations, but in these children the disorder had been given at least 4 different names. Thus, the availability of genetic testing has greatly clarified our understanding of the categories of brain disorders.

**Joubert syndrome.** Another distinctive brain malformation that had resisted gene mapping efforts for many years has also recently been understood at the molecular level, thanks to collaborations between our lab and clinicians in the Gulf region. Joubert syndrome is characterized by a distinctive brainstem malformation (known as the “molar tooth” malformation), and is associated with abnormal breathing, abnormal eye movements, paralysis, and mental retardation, sometimes with autistic symptoms. Previous genetic linkage analysis showed one potential locus on chromosome 9q34, although this locus has not been subsequently confirmed and other linkage screens have been inconclusive. However, 5 families identified primarily from Saudi Arabia allowed us to map a new locus, designated JETS3, and to identify three independent mutations in the AHI1 gene as the cause of Joubert syndrome in these families. Subsequent studies have confirmed the role of AHI1 mutations as a cause of Joubert syndrome in patients from Kuwait and patients from outside the Gulf region as well.

A very important observation scientifically that is illustrated by the AHI1 gene, as well as by some genes associated with microcephaly, is the role that some of the genes associated with human brain development have played in the evolution of the human brain. Our own analysis of AHI1 and AS PM, and analysis by other groups of ASPM and other microcephaly genes, has shown that many of these genes show unmistakable signs that they were the product of positive evolutionary selection on the lineage leading from primates to humans. Presumably, genes that are associated with microcephaly-literally, a small brain-when mutated, may have played a role in the appearance of the large brain size that is so distinctive in humans. On the other hand, the potential evolutionary role of AHI1 is less obvious. Since AHI1 regulates patterns of axon connection, and is essential for coordinated movements of the hands and feet, it might have been involved in the evolution of gait, or the manual dexterity that is also so distinctive of humans.

**Mental Retardation and Autism**
Disorders that are just now becoming accessible to genetic linkage analysis include these disorders, which are both more common, and more potentially treatable, than many of the brain malformations. "Nonsyndromic" mental retardation refers to children who show low cognitive performance but do not show somatic features to suggest a particular "syndromic" cause of mental retardation such as Down syndrome, Fragile X syndrome, etc. These nonsyndromic causes of mental retardation are quite common, especially in the Gulf region. Work on X-linked genes for nonsyndromic mental retardation by our lab and others has shown that many of these genes appear to function predominantly or exclusively in the brain, and often are involved in regulating the function of synapses. They are
potentially attractive targets for development of new therapies, since the brain appears to form relatively normally. More recent work has begun to focus on mapping and identification of autosomal genes for nonsyndromic mental retardation, and again the analysis of the larger families and founder mutations common in Gulf populations has already provided us with the ability to find these important genes. Work is also beginning to focus on children with autistic disorder, a condition that appears to be fairly common in the Gulf region. Autism has been extremely difficult to sort out genetically, presumably because it can reflect mutations in many different genes with similar phenotype. Again, the larger extended families seen in many communities in the Gulf region promise to potentially sort out different genetic causes of autism.

Impact of Genetic Work on Treatment of Childhood Neurological Disorders

Identifying genes for genetically inherited disorders in children has immediate impact in the area of prevention, and is already having major impact on therapeutics as well. For X-linked and recessive disorders, carrier testing can identify those at risk of transmitting the disorder, which for recessive diseases is sometimes useful for premarital DNA testing. But the ability of genetic discoveries in the area of mental retardation to begin to impact therapeutics has been an area of tremendous recent development. So far, these drug trials involve the syndromic forms of mental retardation, which, because of their obvious diagnostic features, have been characterized genetically for 10 years or so. For example, trials of rapamycin, a previously approved drug, are ongoing in a condition called tuberous sclerosis that is associated with mental retardation, autism and seizures. Recent animal work has suggested that HMG co-reductase inhibitors may have efficacy in the neurological effects of mutations that cause neurofibromatosis. And finally, animal work has suggested that lithium carbonate and certain glutamate antagonists may have efficacy in Fragile X syndrome, another important syndromic cause of mental retardation and autism. This work provides an even stronger impetus to understand the genetic causes of mental retardation as fast as possible, because of the burgeoning potential for therapy for the underlying neurological disorder.

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References


Targeting Endothelial Cell Surface Receptors: Novel Mechanisms of Microvascular Endothelial Barrier Transport

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Role of 60kD Albumin-binding Protein in Activating Transcytosis in Endothelial Cells
The delivery of novel biological therapeutic agents across the vascular endothelial barrier (such as monoclonal antibodies targeting specific receptors; e.g., anti-epithelial derived growth factor receptor antibody) represents a major challenge in drug delivery. The situation becomes even more complex in protein delivery across the much more restrictive blood-brain barrier. In studying endothelial barrier function, we have identified new mechanisms of delivery of biologic therapeutics.1-3 Maximum anti-tumor effects or other protective effects of the specific protein thus can be achieved at doses below the maximum tolerated dose. In this review, I summarize below some of key recent development in this area with emphasis on our work.

We and others have investigated the characteristics and function of gp60,4-12 an endothelial cell membrane 60-kDa albumin-binding protein localized in caveolae (2, 5, 6).2,5,6 Gp60 is a crucial protein found on endothelial plasma membrane involved in mediating the flux of albumin across the endothelial barrier by a process termed “receptor-mediated albumin transcytosis”.2,5,6 We have identified the key mechanisms of its activation in regulating endothelial permeability to proteins.5,6 Gp60 organization on the endothelial cell surface is punctate as shown by immunofluorescence using an anti-gp60 antibody conjugated with bifunctional, N-hydroxysuccinimide fluorophore (Cy3).5 Also gp60 was shown to co-localize with caveolae, the plasma membrane invaginations rich in endothelial cells.6 Addition of a secondary antibody to anti-gp60 antibody-treated endothelial cells induced cross-linking of gp60 and resulted in cell surface gp60 clustering or activation.5 This resulted in 3-fold increase in the endothelial cell uptake (or endocytosis) of albumin and the luminal-to-abluminal permeability (or transcytosis) of albumin.5 Importantly, another protein tracer, horseradish peroxidase, was also transported across the endothelial barrier by the engagement of the albumin-mediated transcytosis machinery.5 Thus, these studies showed that macromolecules are transported across the endothelium by a “piggy-back” mechanism in the activation of gp60 (as shown in Fig. 1).

In other studies we used the water-soluble styryl pyridinium dye N-(triethylaminopropyl)-4-(p-dibutylaminostyryl) pyridinium dibromide (FM 1-43) to quantify caveolae-mediated vesicle trafficking across the endothelial barrier by confocal and digital fluorescence microscopy.13 FM 1-43 and fluorescently labeled anti-gp60 antibody were co-localized in endocytic vesicles within 5 min after gp60 activa-
These caveolae-derived vesicles then migrated to the basolateral surface via transcytosis where they released FM 1-43, the fluid phase styryl probe. Also the activation of cell-surface gp60 by cross-linking (as described above) increased trans endothelial albumin permeability. Caveolin-1 and gp60 were shown to co-localize in these vesicles indicating the caveolar origin of the vesicles. Importantly, Src kinase phosphorylation of caveolin-1 was required for the activation of transcytosis and delivery of proteins across the endothelial barrier. Vesicle formation induced by gp60 and migration of vesicles to the basolateral membrane required the interaction of gp60 with caveolin-1 and this was followed by the activation of the Src kinase regulating the signaling of transcytosis. These findings indicate that activation of gp60 secondary to Src activation stimulates transcytosis of proteins across the endothelial cell monolayer. We have demonstrated that this pathway can be exploited for the delivery of protein therapeutics across the vessel wall endothelial barrier (and even possibly the blood-brain barrier).

Identification of Myeloperoxidase-derived Peptide Regulating Protein Transcytosis Across the Vascular Barrier

Another series of studies further advanced the concept of target delivery of therapeutic proteins. We identified the crucial role of an amino acid sequence of myeloperoxidase (MPO) in binding to albumin with high affinity. The binding was shown to be a requirement for the normally high transport of MPO seen across the endothelium. A unique sequence was identified using matrix-assisted laser desorption/ionization (MALDI) analysis of 80- and 60-kDa proteins purified from human lung tissue. These proteins were shown to be the MPO light and MPO heavy chains, respectively. A peptide (RLATE LKSLN PRWDG ERLYQ...
EARKI VGAMVC) corresponding to the MPO-heavy chain (residues 425-454) demonstrated high-affinity binding (in the nanomolar range) to human serum albumin. Replacement of the positively charged residues, R and K, with G prevented the binding of albumin to the peptide,\(^1\) indicating a charge dependent interaction. We observed that albumin increased the binding of an iodinated-MPO tracer to lung microvascular endothelial cells by 2-fold as well as the rate of transendothelial flux of the MPO tracer in vessels,\(^1\) thus indicating the crucial importance of albumin binding to MPO in mediating the transendothelial transport of MPO. Moreover, excess amount of the peptide sequence prevented the interaction between MPO and albumin. Disruption of caveolae with cyclodextrin also prevented the albumin-induced increase in transendothelial flux of MPO indicating the critical involvement of caveolae in this transport mechanism.\(^1\) We observed by confocal imaging that albumin induced the rapid internalization of MPO and its co-localization with albumin-labeled vesicles. MPO was shown to co-localize with the caveolae markers cholera toxin subunit Band caveolin-1 in the endocytosed vesicles. Thus, transcytosis of MPO by caveolae induced by its charge-dependent interaction with albumin is an important means of delivering MPO to the subendothelial space. Moreover, the identified albumin binding peptide sequence of MPO (with its high affinity albumin binding sites) is of great potential clinical significance in the delivery protein drugs across the vascular endothelial barrier in therapeutically relevant dosages (as shown in Fig. 2).

**Clinical Relevance of gp60 and MPO-derived Albumin Binding Peptide to Molecular Therapy and Drug Targeting**

The findings described above have shown the potential exploiting of transcytosis of proteins...
by caveolar trafficking in endothelial cells for the delivery of biological therapeutics across the microvascular barrier. In this context, gp60, the albumin binding protein, is crucial in the mechanism of albumin transport and along with other proteins conjugated to albumin. Gp60 receptor-mediated transcytosis can be exploited for the transport of therapeutically-active agents which do not normally pass through the endothelial barrier.14

Another important clinically relevant drug targeting advance described in the above studies is the discovery of the unique MPO-derived peptide that binds with high affinity to albumin such that albumin-MPO peptide complex can then dock unto gp60 to activate transcytosis.4 This approach is also useful for facilitating the delivery of biologics via the trafficking of caveolae across the vascular barrier.2,3 The identified albumin docking peptide (ADP) sequence of MPO1 can be conjugated to therapeutic proteins such as anti-cancer monoclonal antibodies and injected iv. Thereby ADP-therapeutic protein binds to circulating albumin in a high affinity manner and is transported across the vascular barrier. Thus ADP may be important not only in the delivery of therapeutic proteins but also delivery of diagnostic agents or markers of disease processes to track the efficacy of therapy (e.g., a labeled monoclonal antibody which binds to a receptor marker of a disease). This novel approach has great potential for cardiovascular, cancer, inflammatory, and autoimmune diseases. It may be of value for the treatment of a disease of the CNS (e.g., Alzheimer's Disease, Parkinson's Disease, multiple sclerosis, and amyotrophic lateral sclerosis, and a CNS neoplasia) in which the transcytosis of ADP may facilitate the transport of the therapeutic agent across the blood-brain barrier.

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References


Age-Related Macular Degeneration

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A. Background
Age-related macular degeneration
Age-related macular degeneration (AMD) affects 10 - 20% of people at an age over 65 industrialized nations. Whereas the clinical and the histopathological pictures of AMD are well known, molecular events initiating the disease remain elusive. There is presently no treatment available against the predominant form of AMD, the so-called dry form.

N-retinyl-N-retinylidene ethanolamine
Accumulation of the auto fluorescent age pigment lipofuscin in phagolysosomes of retinal pigment epithelium (RPE) cell is a predictor for the development of the disease. Lipofuscin in the eye harbors two unusual retinoids, the lipophilic cations N-retinyl-N retinylidene ethanolamine (A2E) and its isoform, iso-A2E\(^{1,2}\) (for their structures, see Fig. 1). The compounds can be synthesized \textit{in vitro} from 2 molecules of retinal and 1 molecule of ethanolamine. Both precursors are present in photoreceptor outer segment membranes, where II-cis-retinal serves as the chromophore of the visual pigment rhodopsin, and phosphatidylethanolamine is an abundant membrane phospholipid. \textit{In vivo} A2E is formed in the visual cycle (see

Fig. 1: The structures of A2E and iso- A2E.
Fig. 2: The visual cycle, highlighting the metabolism of retinal in rod outer segments (ROS) and RPE cells.

Fig. 2) presumably from 2 molecules of retinal and 1 molecule of phosphatidylethanolamine. A2E accumulates about ten-fold in the eyes of old compared to young humans and rats.

Mitochondria and apoptosis
The respiratory chain, which transports electrons and protons, is located in the inner mitochondrial membrane and is composed of four complexes (I-IV). At the level of complex IV, which is identical with cytochrome c oxidase, mitochondria reduce molecular oxygen to water and thereby convert energy (Fig. 3). This energy is used to produce ATP and to transport molecules across the inner mitochondrial membrane. Until recently mitochondria were exclusively viewed as life-supporting organelles.

A few years ago it was realized that mitochondria also actively participate in the demise of cells because they are engaged in the execution of apoptosis (see also Fig. 4). Mitochondria contain proteins which, when released into the cytosol, help to selectively de-
stroy vital components of the cell such as enzymes and DNA. Presently four such proteins have been identified: Cytochrome c, apoptosis inducing factor (AIF), SMAC/Diablo, and endonuclease D. Respiring mitochondria have a membrane potential, negative inside, which is the driving force for the uptake of cations into mitochondria. An example is the water soluble Ca2+, which is taken up via a specific pathway. Lipophilic cations are also taken up, even when there is no specific carrier. A classical example is rhodamine, used for staining of mitochondria in living cells. The targeting of mitochondria by lipophilic cations can be devastating, as shown, e.g., with the cation methylphenylpyridinium, which causes Parkinson’s disease.

A2E detaches pro-apoptotic proteins from mitochondria and induces apoptosis in mammalian retinal pigment epithelial cells. We reasoned that the lipophilic cation A2E may be taken up by mitochondria and prompt them to execute apoptosis. We found that A2E at physiologically relevant concentrations induces apoptosis in cultures of all cell types studied so far (six), including RPE cells. A2E-induced apoptosis was accompanied by the appearance of cytochrome c and AIF in the cytoplasm and the nucleus. Biochemical examinations showed that A2E specifically targets cytochrome c oxidase. With both, isolated mitochondria and purified cytochrome c oxidase, A2E inhibited oxygen consumption synergistically with light. Inhibition was reversed by addition of cytochrome c or cardiolipin (1,3-diphosphatidylglycerol, 1,3-DPG), a negatively charged phospholipid, which facilitates the binding of cytochrome c to cytochrome c oxidase. Succinate dehydrogenase activity was not altered by A2E. We suggested that A2E acts as a pro-apoptotic molecule via a mitochondria-related mechanism, possibly through site-specific targeting of this cation to cytochrome c oxidase.

**Light-dependent inactivation of cytochrome c oxidase and prevention by negatively charged phospholipids**

We next investigated the inhibition of cytochrome c oxidase in more detail. The inhibition of cytochrome c oxidase is highly specific for A2E and was observed with the solubilized and reconstituted enzyme. In the dark, 1,3-DPG or other negatively charged phospholipids overcame inhibition. With illumination,
inhibition was stronger, became complete with prolonged exposure, and was thereafter no longer abrogated by 1,3-DPG. Cardiolipin effectively displaced A2E from cytochrome c oxidase, suggesting non-covalent binding of A2E to the enzyme. We concluded that A2E is a potent cytochrome c oxidase-specific inhibitor, which interferes with the binding of cytochrome c to cytochrome c oxidase and, in the light, causes persistent modifications of the enzyme. The modification is probably caused by singlet oxygen, which we found is being produced by A2E in the light.\(^8\)

We also found that the negatively charged phosphatidylglycerol is much more powerful than 1,3-DPG as protector of cytochrome c oxidase in the presence of A2E (Fig. 5).\(^9\) This result is per se interesting but even more important in view of the fact that phosphatidylglycerol is the immediate biosynthetic precursor of 1,3 DPG. Furthermore we found (not shown) that coenzyme Q\(_{10}\) or 1,3-DPG powerfully prevent A2E-induced apoptosis. It is worth mentioning that the level of 1,3-DPG present in old people is only about 50% of that present in young people.

Release of cytochrome c from mitochondria into the cytosol has two major consequences. One is the interruption of electron flow along the respiratory chain. Single electrons are now transferred by components upstream of the cytochrome c binding site to single oxygen...
molecules, which results in superoxide anion formation, oxidative stress, and damage to mitochondria. The other is the stimulation of apoptosis (see above) because of the formation of an "apoptosome" in the cytosol, a pro-apoptotic complex comprising cytochrome c, apaf-1, and procaspase-9. We reported that A2E indeed induces oxidative stress in mitochondria. There is an increased H2O2 and decreased glutathione level in respiring mitochondria exposed to A2E.10 We also found that cytochrome c, AIF, and SMAC/Diablo are released into the extramitochondrial space (Fig. 6).4

Fig. 6: Release of cytochrome c, AIF, and SMAC/Diablo. Time- and A2E concentration-dependence (T, time in min.).

Blue light damage and carotenoids
Excessive light exposure leads to retinal damage, and there is a positive correlation between light exposure and AMD. Blue light is about 50-fold more potent in inducing acute damage than light of the mid-spectral region. Low level chronic exposure produces photoreceptor damage with an action spectrum that peaks at about 500 nm, possibly because of the presence of retinal, A2E, vitamin A, or rhodopsin.

We found that the action spectrum of cytochrome c oxidase inhibition by A2E has a peak around 500 nm (Fig. 7),9 and others have reported a blue light phototoxicity of A2E in RPE cell cultures.11,12 Zeaxanthin and lutein are constituents of the macula lutea. Of the about ten carotenoids found in the blood these two are specifically concentrated in the macula. This is probably not accidental because these dietary carotenoids quench oxygen free radicals and singlet oxygen, and they act as blue light filters. A high intake of food rich in lutein and zeaxanthin is correlated with a lowered risk for AMD, and a low plasma level is associated with a higher risk for AMD.

Is AMD a “mitochondrial disease”? In recent years it became apparent that many diseases are caused by malfunctioning mitochondria.13 The specificity of A2E's action at the level cytochrome c/1,3-DPG/cytochrome c oxidase allows the proposal14 that AMD is a "mitochondrial disease", i.e., a disease which is caused by malfunctioning...
mitochondria. If this were the case, AMD should have a higher prevalence in patients who suffer from already identified mitochondrial diseases such as myopathies or maternally inherited diabetes and deafness. There is evidence in the literature\textsuperscript{14} that this is indeed so.

**B. From Basic Research to Medical Application**

Loss of RPE cell viability through inhibition of mitochondrial functions is highly likely to constitute a pivotal step towards the progressive degeneration of the central retina. We proposed earlier \textsuperscript{10,14} to prevent or cure AMD on the basis of the knowledge outlined above. In very general terms, we now propose to use food additives or eye drops containing negatively charged phospholipids, and/or carotenoids, and/or antioxidants.

**Conclusion**

The lipophilic cationic pigment A2E, which is formed in the eye particularly in old individuals and is excited by blue light causes apoptosis.
by specifically interacting with mitochondria. The primary action of A2E is the prevention of cytochrome c binding to cytochrome c oxidase and, in the light, a persistent inactivation of cytochrome c oxidase. This is followed by release of pro-apoptotic proteins and oxidative stress. Negatively charged phospholipids counteract A2E and prevent apoptosis, as does the antioxidant coenzyme Q10. (Fig 8).

The identification of the mode of A2E’s action suggests promising strategies to prevent or reverse AMD.

References


Hypertension, with a prevalence of up to 30% throughout the world, is increasing in incidence in more affluent and aging populations. Because it is totally asymptomatic, it has been named the "silent killer," as it is the major contributor-or risk factor-to cardiovascular morbidity and mortality. A large body of research over the past several decades has been devoted to investigating the causes and mechanisms of hypertension. Although the causes-genetic and environmental-remain obscure, much progress has been made in elucidating some of the pathogenic mechanisms causing hypertension, as well as its common complications, ie. ischemic heart disease, stroke and renal failure. The ultimate goal of this research is to inhibit these mechanisms and thus prevent or reverse the hypertensive complications.

Most prominent among these mechanisms are the renin-angiotensin system (RAS) and salt. Renin was discovered at the end of the 19th century by Tigerstedt and Bergmann and the components of the RAS had been characterized earlier in the 20th century through the work of Braun-Menendez in Argentina and Page in the United States. However, there were doubts on the importance of the RAS in the pathogenesis of hypertension and common hypertensive complications, mainly because of failure to find a correlation between levels of renin or angiotensin II (Ang II) and levels of blood pressure. A series of experimental and clinical studies starting in the 1970's helped clarify this apparent paradox by demonstrating the reciprocal relationship between sodium balance and RAS status. It was proven that an activated RAS causes not only hypertension, but also severe tissue damage in vital organs and that blockade of the RAS - either via Ang II receptor antagonists or angiotensin-converting enzyme (ACE) inhibitors - can prevent or reverse end-organ damage.

It was shown that exogenous Ang II excess in experimental animals1 or endogenous Ang II stimulation in humans2 resulted in severe myocardial necrosis and scarring, as well as renal tubular necrosis leading to renal failure, not necessarily correlated to blood pressure levels. A parallel survey of a hypertensive population revealed that high-renin hypertensives had a significantly higher rate of cardiovascular complications than low-renin hypertensives.3,4 Further work elucidated the close reciprocal relationship between the RAS and salt - the other major mechanism of hypertension. It was shown that both a RAS-mediated vasoconstriction and a salt-mediated mechanism contribute to development and maintenance of hypertension. Salt overload is associated with suppressed renin levels, but when salt is removed (via diuretic treatment and/or low-salt diet) the RAS becomes activated and partly offsets the benefits of diuretic therapy.5-7 At the time, the prevailing opinion was that salt-mediated hypertension was due to retention of fluid and expansion of intravascular fluid volume (despite evidence of contracted...
blood volume and increased peripheral vascular resistance in most low-renin patients). However, subsequent experimental studies reconciled these findings by demonstrating that sodium excess stimulates initially vasopressin and later the sympathetic nervous system, which sustains the peripheral vasoconstriction.8-12 Therefore, the salt-dependent hypertension may indeed be "volume expanded" in terms of extracellular (but not intravascular) fluid volume, but is still characterized by increased arteriolar resistance.

Later experimental and clinical work demonstrated that interference with mechanisms of sympathetic activation at various levels, including pharmacologic sympathetic blockade, as well as genetic engineering or gene treatment to obliterate selected adrenergic pathways, can totally abolish the hypertensive response to excessive salt loading, both acute (infused parenterally) and chronic (dietary).13-16 This provided a final proof that the mechanism of salt-dependent hypertension is vasoconstriction due to sympathetic excitation and not hydrostatic pressure due to expanded plasma volume - which should not be surprising, as conditions with documented expanded plasma volume without salt retention, such as the syndrome of inappropriate antidiuretic hormone (SIADH) release, are characterized by hemodilution but not elevation of systemic blood pressure.

The elucidation of RAS-mediated mechanisms for the pathogenesis of hypertension and end-organ hypertensive damage, such as ischemic heart disease, heart failure, renal failure and stroke, was soon followed by development of therapeutic approaches to prevent or reverse these conditions. The earliest studies demonstrating therapeutic benefits of RAS blockade in hypertension and heart failure were conducted with pharmacologic probes given parenterally, such as the Ang II antagonist saralasin17,18 and the ACE inhibitor teprotide.19,20 The encouraging results of these pioneering studies led the pharmaceutical industry to develop first orally active Ang II type 1 receptor blockers (ARBs).23 Both these classes of drugs have now become recommended standard therapy for hypertension, heart failure, ischemic heart disease, chronic kidney disease (particularly diabetic nephropathy) and cerebrovascular disease.24 A vast number of long-term randomized clinical outcome trials have provided ample evidence that ACEi-based or ARB-based therapy can prevent, reverse or at least decelerate the progression of cardiovascular morbidity and diminish mortality.

In parallel, significant progress has been made in the elucidation of sodium triggered mechanisms for the pathogenesis of salt-induced hypertension. It has now been demonstrated that acute or chronic salt-excess alters the function of the α2 adrenergic receptors (ARs) in the central nervous system. The data suggest that elimination of the sympato-inhibitory α2A-AR subtype or activation of the sympato-excitatory α2B-AR subtype leads to hypertension. Conversely, activation of the α2A-AR subtype has a hypotensive effect and elimination of the α2B-AR subtype abolishes the hypertensive response to salt-loading.13-16, 25,26 These more recent findings have not yet produced practical therapeutic approaches for selective treatment of the hyperadrenergic state that characterizes certain forms of hypertension, decompensated heart failure, coronary disease, arrhythmias, autonomic imbalance, etc. Nevertheless, a better understanding of the role of specific AR-subtypes in the pathogenic mechanism of these conditions should eventually lead to development of appropriate therapeutic interventions, as excessive dietary salt intake is probably the most important environmental factor causing hypertension in people who either are genetically salt sensitive or become so as a consequence of aging.

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The Genetic Research Cycle in Human Disease: The Huntington’s Disease Paradigm

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Abstract
For more than a century after the description of Huntington’s Disease (HD), an inherited neurodegenerative disorder, by George Huntington, a physician on Long Island New York, research into the affliction concentrated on clinical and neuropathological correlates, trying to work backwards from these phenotypes to crucial steps earlier in the disease pathway. These efforts provided excellent clinical and pathological descriptions, and supplied chemical models in rodents, but working backwards from the ultimate disease phenotype did not identify definitive, causative biochemical steps in the disease process or provide a rational route to an effective therapy. In 1983, an alternative approach emerged, aimed at studying pathogenesis from its starting point, the HD gene, rather than from its endpoint, neuronal dysfunction and loss. This paradigm, which I term the Genetic Research Cycle, has acted as a model for similar investigations in a wide range of neurological disorders, leading to better diagnosis and patient management, deeper understanding of pathogenesis, and routes for developing rational, effective treatments.

The Genetic Research Cycle
The Genetic Research Cycle in human disease begins and ends with human patients. In its first phase, phenotypic information from the patient population is combined with the rules of genetics to first map and then to identify DNA sequence variations that produce or modify the disease phenotype. The next phase focuses on determining the mechanism by which the difference in DNA sequence leads to the observed phenotype. This is generally investigated in model systems via biochemistry, cell biology, structural biology, mouse or lower organism modeling. However, it is critical to be guided in this step by genotype-phenotype relationships exhibited by human patients to ensure that the correct mechanisms are being studied. Finally, in the last phase of the cycle the understanding gained concerning the disease process feeds back as benefit to the patient population in the form of better diagnosis and management and more effective, rational treatments. We are now in this final phase in Huntington’s Disease (HD), which has acted as a model to promote application of this strategy in a wide variety of both common and rare disorders.

Huntington’s Disease
HD, with a prevalence of 1 in 10,000, is a severe late-onset dominantly inherited neurodegenerative disorder that is characterized by progressive involuntary choreiform (dance like) movements, psychological disturbance,
cognitive decline and a distinctive pattern of neuropathology. It typically has its onset in mid-life, though it may occur in juveniles or the elderly, and produces an inexorable decline to death. The initial signs of motor disturbance are slight, such as awkwardness of gait, clumsiness, facial twitching or subtle involuntary movements of the fingers. Progression makes the involuntary movements more frequent and exaggerated, impairing and eventually eliminating normal day-to-day activity by interfering with the ability to walk, stand, write, speak, and swallow. In some cases, particularly those of juvenile onset, extreme rigidity is seen rather than chorea. Emotional and behavioral changes often accompany or precede the onset of movement disorder and can have a profound impact on ability to function and on interaction with family members. Impulsive, erratic behavior, impaired memory, poor concentration, moodiness and chronic depression can all be early signs of HD. The distinctive symptoms are due to loss of neurons that is most prominent among medium spiny projection neurons in the striatum but is also seen more diffusely in the cortex. After 10-20 years of decline that leads to severe emaciation and incapacitation, the afflicted individual usually succumbs to heart disease, choking or pneumonia secondary to aspiration. There is currently no effective treatment for preventing the onset or delaying the progression of HD, but hope that a rationale therapy may be on the horizon has come from more than two decades of molecular genetic analysis.

Discovering the Cause of HD

In 1983, we mapped the HD gene to a chromosomal position, without any knowledge of the nature of the genetic defect, by tracking the segregation of polymorphic DNA markers in families with the disorder. Statistically significant coinherence with the marker G8 (D4S10) in large HD families, in particular an extremely large kindred from Venezuela provided by Nancy Wexler and her clinical colleagues, indicated that the HD gene lies near the short arm telomere of the 4th chromosome. This landmark study was the first successful application of this nascent technology and it triggered a flood of similar investigations that has led to the mapping of hundreds of other human disease genes, including those in important neurological disorders like Alzheimer disease, Parkinson disease and amyotrophic lateral sclerosis, among many more. In HD, the marker discovery introduced the capacity for predictive diagnosis decades before "at risk" individuals showed any abnormal signs. It gave HD family members the option to learn their HD gene status, eliminate the uncertainty and better plan their lives and, more importantly for many, offered prenatal diagnosis to ensure that they did not pass the killer gene on to their offspring.

The marker discovery also acted as the foundation for a decade-long quest to isolate and characterize the genetic defect based on its chromosomal location, which culminated in 1993 in the identification of an expanded, unstable CAG trinucleotide repeat in the 5'-coding sequence of the gene encoding huntingtin, a 350 kD protein of unknown function. The discovery of the disease gene provided a direct diagnostic test for HD and signaled the beginning of a new effort to delineate the pathway of pathogenesis beginning with its initial trigger, the HD mutation.

Genetic Criteria for the Mechanism that Initiates HD Pathogenesis

For the past dozen years, the question of how the expanded CAG repeat triggers the characteristic pattern of neuronal death in HD has focused on a 'gain-of-function' in which a novel property is conferred on the protein. The HD mutation does not simply eliminate the normal function of huntingtin, as individuals with one copy of the gene inactivated by deletion or translocation do not display HD-like symptoms. Indeed, the effect of the expanded CAG stretch must be completely dominant to explain the similar age at onset in HD heterozygotes and HD homozygotes.
Huntingtin is expressed in a wide variety of cells in both the nervous system and peripheral tissues, in contrast to the specific pattern of cell death in HD. The expanded CAG stretch of the disease allele does not eliminate huntingtin expression, but alters its structure by extending a stretch of polyglutamine near the amino-terminus, conferring a new property that is peculiarly detrimental to striatal neurons. Several other neurodegenerative disorders with different patterns of neuronal loss, including spinal-bulbar muscular atrophy, dentatorubropallidoluysian atrophy, and spinocerebellar ataxias 1, 2, 3, 6, 7 and 17, have also been found to be caused by expanded polyglutamines in different proteins. All of these disorders and HD may share a common mechanism of neuronal toxicity in which the susceptibility of particular neuronal populations is determined by the context in which the lengthened polyglutamine segment is presented.5

Genotype-phenotype comparisons in HD and other polyglutamine disorders have led to several genetic criteria describing the mechanism that triggers pathogenesis5,7: 1) a threshold polyglutamine length below which the disease does not occur in a normal lifespan; 2) progressive severity with increasing polyglutamine length above the threshold; 3) dominance over the normal copy of the gene present in most heterozygous HD patients; 4) relatively greater sensitivity to increasing polyglutamine length than to the increased disease protein concentration produced from two disease alleles in homozygotes; and, most importantly, 5) specificity of neuronal loss that is influenced by the nature (structure, function or localization) of the protein containing the polyglutamine tract. These criteria have provided a means for judging potential mechanisms and have led to the notion that the deleterious property of the polyglutamine tract in HD may involve an altered conformation of huntingtin’s amino terminal region.

Modeling the HD Pathogenic Trigger

The existence of a novel conformational property of mutant huntingtin has been supported by in vitro studies of a small amino-terminal huntingtin fragment, where an expanded polyglutamine tract promotes self-aggregation with a conformational change of the polyglutamine tract from a random coil to an amyloid structure.8,9 Aggregation may also occur in vivo, as aggregated material has been detected in post-mortem HD brain and the first transgenic HD mouse model, which expresses an amino-terminal fragment of mutant huntingtin, develops large intranuclear aggregates in neurons and many other cells. However, the huntingtin fragment mouse model does not show neuronal loss comparable to HD, indicating that amino terminal fragment is not sufficient to trigger the pattern of HD pathogenesis seen in humans.

In a precise genetic model of the human HD patient, knock-in mice in which the HD mutation has been introduced into the mouse orthologue, Hdh, significant biochemical and histological phenotypes are associated with expression of full-length mutant huntingtin at normal physiological levels and in a normal developmental pattern. These abnormalities occur first in the striatum, and are evident many months before any huntingtin fragment or aggregates are seen, indicating an ongoing pathogenic process that only much later leads to huntingtin fragment and consequent aggregates. The early full-length huntingtin phenotypes fulfill all of the genetic criteria defined from human patients, including striatal specificity, suggesting that they result from the same pathogenic trigger as the human disorder, an altered conformational property of the amino terminus acting within full-length mutant huntingtin rather than within a fragment. Unlike its behavior in a small fragment, the effect of the novel conformational property in full-length huntingtin does not directly promote aggregation but may alter huntingtin’s interactions with other
cellular elements. We have argued previously that the aggregation promoting property observed in experiments with amino terminal fragment may act as a proxy for the conformational property in full-length protein. Consequently, we have identified a number of small molecules that inhibit in vitro aggregation of the amino-terminal huntingtin fragment. As predicted, some of these reverse a full-length mutant huntingtin phenotype in cultured striatal cells, suggesting that the identification of compounds that attack the abnormal conformational property of mutant huntingtin is a viable approach to identifying potential therapeutics.

Completion of the Genetic Research Cycle and Starting Again

With the advent of large-scale drug screens to alter gene-based phenotypes, the HD field is on the brink of large scale clinical trials that will test potential treatments designed to interfere at various stages of the pathogenic process, including its very beginning. The delineation of an effective treatment would complete the Genetic Research Cycle in this disorder. However, we are already taking the cycle through a second round, this time looking at using genetic strategies for finding gene modifiers of the HD phenotype. By identifying genetic factors that influence age at onset of HD, we aim to provide prevalidated targets for novel drug development that are already guaranteed from human patient studies to alter pathogenesis.

Conclusion

While the Genetic Research Cycle is nearing completion in HD, the model that it provided has been applied widely in neurological disorders, and each of these is also moving around the cycle, with the hope for better diagnosis, management and treatment at its end. The new tools being generated by advances in human genomics, such as the HapMap, should not only add the capacity to pursue the cycle in complex disorders, but will also accelerate the rate with which each turn is completed. Genetic strategies have already changed the practice of medicine in neurological disorders, and that change, to the benefit of the patients and their families, is likely to continue at an even more rapid pace.

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