Scientific Side Trips:
Six Excursions from the
Beaten Path

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Origin of a 40-Year Research Partnership

Our 40-year research partnership began on a sunny June day in 1966 at the Massachusetts General Hospital, where we met with twelve other nervous neophytes to begin our internships in internal medicine. Within a few days, we developed the friendship and mutual respect that were to sustain our collaborative effort over four decades. Our backgrounds were quite different. Joe grew up in small-town South Carolina and graduated from the young University of Texas Southwestern Medical School in Dallas, where he came under the spell of Donald W. Seldin, chairman of medicine and intellectual father to generations of physician-scientists. In stark contrast, Mike grew up in big-city Philadelphia and graduated from the nation’s oldest medical school, the University of Pennsylvania School of Medicine. We were drawn together by a shared fascination with clinical medicine and medical science and a desire to one day make discoveries of significance to both.

In 1968, after two years of residency, we were both accepted for scientific training at the National Institutes of Health (NIH), where Joe worked in the laboratory of Marshall Nirenberg, who was soon to receive a Nobel Prize for solving the genetic code. Mike worked with Earl Stadtman, the biochemist’s biochemist who later received the United States National Medal of Science. We also had clinical duties. Joe was assigned to care for a pair of siblings (ages 6 and 8) who were suffering repeated myocardial infarctions due to massively elevated levels of low-density lipoprotein (LDL) cholesterol in their blood. The diagnosis was homozygous familial hypercholesterolemia (FH), a rare form of a common genetic disease, the pathogenesis of which was unknown. The two of us discussed these children intensely, and we resolved some day to unlock the genetic secret behind this striking illness.

In 1970, after two years at the NIH, Joe moved to Seattle to learn medical genetics under the skillful tutelage of Arno Motulsky. There, Joe spearheaded a classic study of the common Mendelian causes of hyperlipidemia in survivors of myocardial infarction. Mike remained one more year at the NIH. In 1971, he moved to the University of Texas Southwestern Medical Center, where he completed a fellowship in gastroenterology and began to study the properties of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), the rate-controlling enzyme of cholesterol synthesis that was later shown by Akira Endo to be the target of the statin drugs (Ref. 1; see Ref. 2). Mike’s move to Dallas was strongly advocated by Joe, who enlisted the aid of Seldin. Mike’s wife, Alice, a born-again Yankee, agreed to the move, but only under the condition that they would stay for one or two years at most. That was four decades ago, and the couple is still happily in residence, having raised two Texan daughters.

In 1972, Joe returned to Dallas, and we began our collaborative studies of FH. This work led to the discovery of the LDL receptor, the process of receptor-mediated endocytosis, and the mech-
anism by which LDL receptors control the level of cholesterol in blood. All of this early work (1972–1985) has been reviewed elsewhere, most notably in our Nobel lecture, which was published in Science in 1986 (3). It has also been detailed in more recent historical reviews (4, 5).

In 1993, our study of the regulation of LDL receptors led to the discovery of sterol regulatory-element binding proteins (SREBPs), transcription factors that induce lipid synthesis and uptake in animal cells (6–9). The crucial purification was performed by two postdoctoral fellows, Xiaodong Wang and Michael Briggs, and the cDNA cloning was accomplished by Chieko Yokoyama, a postdoctoral fellow, and Xianxin Hua, a graduate student. The novel feature of SREBPs is that they are synthesized as intrinsic membrane proteins of the endoplasmic reticulum (ER), and they must be transported to the Golgi complex, where they are cleaved to send active fragments to the nucleus. We described this transport process and its feedback regulation in detail and named it regulated intramembrane proteolysis (RIP). Like receptor-mediated endocytosis, RIP turned out to be a fundamental biologic mechanism that is used in more than forty other regulatory systems, including the proteolytic processing of the developmental protein Notch, the stress protein ATF6, and the amyloid precursor protein, which is cleaved by RIP to form the pathologic amyloid-β peptide (10, 11). Earlier review articles detail the combined genetic and biochemical approaches that we used to delineate the transport proteins and proteases responsible for RIP of SREBPs and to show how these cleaved transcription factors activate the complete program of cholesterol and fatty acid synthesis in the liver (12–15).

In this Reflections article, we will not discuss LDL receptors, SREBPs, or cholesterol homeostasis. Rather, we focus on six side projects that we pursued over the years, each affording an adventurous diversion from the central core of our research and each teaching us a principle useful to science and medicine. In describing each project, we credit the students and postdoctoral fellows who were most responsible. However, we do not mean to slight the many others who made important, even crucial, observations. We value all of them. Similarly, in citing work from other laboratories, we focus on the papers that stimulated us or expanded on our work. This is a very personal list, and we apologize in advance to the many unnamed scientists who made substantial contributions to each field. This article represents a personal reflection and not a comprehensive review.


Our discovery of scavenger receptors began with a pathogenic paradox. Patients with homozygous FH lack LDL receptors; therefore, their cells cannot take up LDL, yet the same patients accumulate massive amounts of LDL-derived cholesterol in scavenger cells of the body, primarily macrophages. Moreover, even in subjects with normal LDL receptors, it is impossible to overload macrophages with cholesterol by incubation with LDL. When macrophages, or any other cells, begin to accumulate cholesterol, LDL receptors are reduced by feedback inhibition of SREBP processing, yet LDL cholesterol accumulates to high levels in macrophages within atherosclerotic plaques, converting them into foam cells. Clearly, macrophages must have an alternate, non-suppressible pathway to take up LDL.

In 1978, together with Y. K. Ho and Sandip Basu, our first two postdoctoral fellows, we isolated macrophages from mouse peritoneal fluid and incubated them with native LDL (16). As expected, the cells did not accumulate excess cholesterol because their LDL receptors became down-regulated (Fig. 1A). We had earlier shown that acetylation of lysine residues on LDL destroys its ability to bind to LDL receptors, and we wondered whether the acetyl-LDL would have acquired the ability to be taken up by macrophages. To our delight, this was the case (Fig. 1B). Macrophages expressed a high-affinity receptor that bound and internalized acetyl-LDL without any down-regulation. The excess cholesterol was esterified and stored in the cytosol as cholesteryl esters, whose concentration was increased by 40-fold in the cells, converting them to classic foam cells.

Remarkably, the macrophage receptor was not specific for acetyl-LDL. Other negatively charged macromolecules, including maleyl-LDL, maleyl-albumin, sulfated polysaccharides (such as fucoidan and dextran sulfate), and polynucleotides (such as poly(I) and poly(G)) competed for acetyl-LDL uptake. There was some selectivity, however. Certain negatively charged polymers (such as poly(C), poly(A), and poly(β-glutamic acid)) did not com-
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neglected to consider that our buffer contained EDTA, by macrophages. The result was negative. We had overnight, and the next day, we tested the LDL for uptake with buffer. We bubbled oxygen through the solution put human LDL in a dialysis bag immersed in a flask filled idues, giving the LDL a negative charge. Accordingly, we considered that LDL might undergo oxidation, and some of the oxidation products may have attached to lysine res-
sible physiologic ligand for the scavenger receptor, we pos
tor. This receptor activates transcription of a variety of genes (including those for apoE and ABCA1) that are required an external cholesterol acceptor to excrete the stored cholesterol from the cell. When we added an external acceptor, such as high-density lipoprotein (HDL), the stored cholesteryl esters were hydrolyzed by a cytosolic neutral lipase, and the cholesterol was released to the acceptor. HDL was not the only acceptor in blood. Indeed, native LDL could serve as an acceptor, and so could other plasma proteins, such as thyrogbulin, but surprisingly not albumin. The most potent acceptors were membranes prepared from red blood cells, suggesting that red blood cells may be active participants in the shuffling of cholesterol between organs (20). To our knowledge, this hypothesis has not been tested, but it may be important in the process that is now known as reverse cholesterol transport.

We also observed that addition of acetyl-LDL to macrophages stimulated the cells to produce apolipoprotein E (apoE), which can target lipoproteins for uptake by the liver (21). This finding presaged the later observations of Peter Tontonoz, Peter Edwards, and David Mangelsdorf, who showed that cholesterol loading of macrophages leads to activation of the nuclear hormone liver X recep-
tor. This receptor activates transcription of a variety of genes (including those for apoE and ABCA1) that are designed to release stored cholesterol from macrophages (22, 23).

We have one regret about the macrophage studies, and it involves missing oxidized LDL. In thinking about a possible physiologic ligand for the scavenger receptor, we considered that LDL might undergo oxidation, and some of the oxidation products may have attached to lysine residues, giving the LDL a negative charge. Accordingly, we put human LDL in a dialysis bag immersed in a flask filled with buffer. We bubbled oxygen through the solution overnight, and the next day, we tested the LDL for uptake by macrophages. The result was negative. We had neglected to consider that our buffer contained EDTA, which binds the trace metals that are needed for the lipid oxidation reaction. Daniel Steinberg and colleagues did the right experiment and found that oxidized LDL did indeed bind to the macrophage receptor (24, 25), and Alan Fogelman and colleagues showed that oxidation of unsatu-
ated fatty acids in LDL generates malondialdehyde, which forms Schiff bases with lysines, removing the positive charge and mimicking acetylation (26). Although definitive proof is still lacking, the accumulated evidence suggests that LDL becomes oxidized in atherosclerotic plaques, and this causes it to bind to scavenger receptors, creating the foam cells that are the hallmark of this disease (27). Antibodies to oxidized LDL may prove to be useful in blocking macrophage cholesterol accumulation and pre-
venting myocardial infarctions (28).

In 1990, the acetyl-LDL receptor was purified and its cDNA was cloned by our former postdoctoral fellow Monty Krieger and his postdoctoral fellows at the Massachusetts Institute of Technology (29). Now called SR-AI (for scavenger receptor class A, type I), the original scavenger receptor has been joined by a family of twelve other structurally diverse membrane receptors, all of which take up acetyl-LDL and/or oxidized LDL. They also bind and internalize a wide range of other ligands, including lipid molecules displayed on the surface of bacterial pathogens and modified self-molecules displayed on apoptotic cells. The extensive literature on the roles of scavenger receptors in innate immunity, microbial pathogenesis, and various pathologic processes (in addition to atherosclerosis) has been described nicely in a series of review articles by Siamon Gordon and colleagues (30, 31).


We became interested in protein prenylation when we read the papers of John Glomset and co-workers, who showed that certain proteins in animal cells possess hydrophobic prenyl groups, 15-carbon farnesyl or 20-carbon geranylgeranyl attached to cysteines in thioether link-
age (32). Prominent farnesylated proteins include Ras pro-
teins, whose activating mutations are among the most common causes of cancer. Remarkably, Glomset’s find-
ings were made possible by Akira Endo’s discovery of HMG-CoA reductase inhibitors (1). Thus, a discovery aimed toward heart disease increased our fundamental knowledge of cancer. So much for categorical NIH institutes!

All prenyl groups are derived from mevalonate, which is produced by HMG-CoA reductase, the rate-controlling
enzyme in the cholesterol biosynthetic pathway (33). Glomset’s discovery was triggered by his observation that blocking HMG-CoA reductase with a statin precluded cells from entering the S phase of the cell cycle. Adding cholesterol did not reverse this deficiency, suggesting that the cells needed another product derived from mevalonate. While blocking production of endogenous mevalonate with a statin, Glomset labeled the cells with [3H]mevalonate and found incorporation into several proteins (32). Work in yeast and animal cells soon showed that one set of prenylated proteins always terminates in the amino acid sequence CAAX, where A stands for aliphatic, and X can be one of several residues (34, 35). The X residue determines whether a protein will be modified with a farnesyl or a geranylgeranyl. Oncogenic Ras proteins are farnesylated, whereas structural proteins like Rho and Rac are geranylgeranylated (36). After prenylation, the proteins are cleaved by a protease, so the prenylated cysteine becomes the COOH-terminal amino acid, and its free COOH group is then methylated, removing all charges and allowing the prenyl group to insert into membranes. Another class of geranylgeranylated proteins called Rab proteins does not contain a CAAX box. Geranylgeranylation occurs on one or two cysteines near the COOH terminus.

The findings of Glomset and the yeast geneticists fascinated us because of our longstanding interest in the mevalonate pathway, and we were intrigued by its potential connection to cancer (33). At this point, we were joined by Yuval Reiss, a postdoctoral fellow from Israel who had worked on ubiquitination with Aaron Ciechanover and Avram Hershko and had mastered the technique of affinity chromatography. Yuval made an affinity column with a CAAX peptide and used it to purify farnesyltransferase from rat liver. The enzyme was a heterodimer composed of an α-subunit that had the transferase activity and a β-subunit that bound to the Ras substrate (37, 38). Yuval’s enzyme was efficient in farnesylating Ras proteins. Most importantly, the enzyme could be inhibited by CAAX peptides as short as four residues (37, 39). Others had shown that farnesylation is essential for the transforming activity of mutant oncogenic Ras proteins. Together with Guy James, a postdoctoral fellow, we immediately began a collaboration with Genentech chemists in an effort to design peptidomimetics that would enter cancer cells and block Ras prenylation (40).

Ras proteins come in three varieties. H-Ras is the most heavily studied, but it is not the one that is frequently mutated in human cancers. That distinction belongs to K-Ras, which has a polylysine sequence immediately upstream of the CAAX box. The polylysine sequence gives K-Ras a 50-fold higher affinity for the farnesyltransferase compared with H-Ras (41). James Marsters and his colleagues at Genentech designed a series of benzodiazepine peptidomimetics that were extremely efficient in blocking farnesylation of H-Ras. Unfortunately, these compounds were much less effective in blocking farnesylation of K-Ras. Even more disappointing, we found that when its farnesylation was blocked, K-Ras became an alternative substrate for another enzyme that we characterized, namely geranylgeranyltransferase (now known as GGTase I) (Ref. 41; see below). Realizing that it would be difficult to prevent all prenylation of K-Ras, we reluctantly gave up our quest to cure cancer. Others persisted, but the clinical results have been disappointing, and currently, there is no approved farnesyltransferase inhibitor in clinical use.

Our search for a geranylgeranyltransferase began with the observation by others that geranylgeranyl, not farnesyl, was added to proteins with CAAX boxes terminating in leucine. Chief among these are Rac and Rho proteins that anchor cell membranes to the cytoskeleton. Like Ras proteins, Rho and Rac are GTP-binding proteins. At this point, Yuval Reiss was joined by Miguel Seabra, a graduate student from Portugal. Together, they purified the enzyme that became known as GGTase I. Remarkably, we found that farnesyltransferase and GGTase I share the same catalytic α-subunit. They differ in the β-subunit, which confers the specificity for Ras or Rac/Rho proteins, respectively (42).

Miguel Seabra went on to make a discovery that was even more intriguing. He set out to purify a third prenyltransferase, namely the enzyme that attaches geranylgeranyl to Rab proteins, a large family of GTP-binding proteins that regulate vesicle fusion reactions. Because Rab proteins do not contain CAAX boxes, Miguel had to use standard column purification techniques. Surprisingly, when he applied a partially purified enzyme preparation to a hydrophobic column, none of the activity was recovered in the eluate. When this happens, the standard approach is to mix together the eluate fractions just in case the enzyme contains two necessary subunits that were separated on the column. This type of mixing experiment almost never works. Nevertheless, we urged Miguel to mix the fractions. All of us were delighted by the result. Indeed, the column had separated two required components, which we called Components A and B (43). With this knowledge, Miguel was able to complete the purification of both components, always assaying one component in the presence of the other. Component B contained two polypeptides that
roughly resembled in size the α- and β-subunits of farnesyltransferase and GGTase I. Component A was unique (44). Miguel Seabra and Doug Andres, a postdoctoral fellow, then performed a series of kinetic studies that defined the separate roles of Components A and B. It turned out that Component B is indeed the catalytic component (45). On its own, Component B attaches a single geranylgeranyl to a single Rab protein. In the absence of Component A, the reaction terminates at that point because the geranylgeranylated Rab remains bound to the enzyme. When Component A is added, it removes the geranylgeranylated Rab from the enzyme and allows the enzyme to undergo further rounds of catalysis. We thus renamed Component A as Rab escort protein (REP), later called REP-1. Its function is to bind the hydrophobic geranylgeranyl group on the Rab protein, shielding it from water and allowing it to insert subsequently into the correct vesicular membrane. CAAX box proteins do not need an escort because the prenyl group is added while the COOH-terminal residue retains its hydrophilic negative charge.

We obtained the sequence of six tryptic peptides from the REP-1 component of Rab GGTase, and when we blasted them through gene databases, we got an enormous surprise. The comparison predicted that rat REP-1 is the orthologue of a human gene that had been implicated in an X-linked retinal disease called choroideremia, which causes blindness in young boys (44). We obtained cultured lymphoblasts from four boys with choroideremia and found that the cell extracts were severely deficient in transferring geranylgeranyl to Rab proteins. Activity could be restored by adding back purified REP-1, but not Component B, confirming that the disease is caused by REP deficiency (46).

Turning from biochemistry to molecular biology, Doug Andres, Wen-Ji Chen (postdoctoral fellow), and Scott Armstrong (M.D./Ph.D. student) cloned cDNAs for the subunits of all three prenyltransferases (45, 47–49). Fig. 2 illustrates their subunit composition. When Doug cloned the cDNA encoding REP-1, he confirmed that the choroideremia gene encoded REP-1 (45). Although REP-1 is expressed in all mammalian cells, its deficiency causes only retinal degeneration. As shown by Scott Armstrong and Frans Cremers (postdoctoral fellow), other cells produce a close relative of REP called REP-2, which substitutes for a deficiency of REP-1 (50). Recently, we became aware of a gene therapy trial in which a virus encoding REP-1 is injected into the eyes of boys with this condition. Following successful preclinical studies in a mouse model of choroideremia (51), the first such patient was injected in Oxford, United Kingdom, in October 2011.1

Our diversion into prenyltransferases did not cure cancer, but it did contribute to our understanding of the enzymology of protein prenylation and may eventually lead to a cure for a rare form of blindness. If anything, this excursion exemplifies in vivid fashion how curiosity-driven basic science can have unanticipated positive consequences for medicine.


Monocarboxylate transporters (MCTs) facilitate the bidirectional movement of short-chain carboxylic acids, most prominently lactate and pyruvate, into and out of cells. Their presence had been inferred for many years based on kinetic studies of monocarboxylate uptake, primarily in red blood cells, but their molecular identities were unknown.

By pure accident, we isolated the first cDNA for a MCT, giving birth to a new family that now has fourteen members. The story begins with studies of the uptake of mevalonate, which is not normally a substrate for any MCT. In 1981, Monty Krieger, then a postdoctoral fellow in our laboratory, developed a method to isolate Chinese hamster ovary (CHO) cells with mutations in the gene for the LDL receptor (52). After he joined the faculty at Massachusetts Institute of Technology, Krieger and his student David Kingsley used this approach to isolate a mutant cell line, designated ldLA-7, which failed to take up LDL because of a deficiency of LDL receptors (53).

Working with Jerry Faust, formerly a long-term research associate in our laboratory, Krieger attempted to correct the LDL receptor deficiency by transfecting the

1. M. Seabra, personal communication.
cells with a cDNA library prepared from wild-type CHO cells. They hoped that this approach would lead to the isolation of the LDL receptor gene. They grew the cells in a medium called MeLoCo, which was designed to render cells totally dependent on the LDL receptor for growth. The medium contained the HMG-CoA reductase inhibitor compactin to block endogenous mevalonate synthesis, thereby forcing the cells to rely on LDL uptake to meet their cholesterol requirement. The medium also contained a low concentration of mevalonate, sufficient to supply the small amount of mevalonate needed for trace non-sterol products like prenylated proteins but not sufficient to meet the cell’s bulk cholesterol need. Lacking LDL receptors, the IdIA-7 cells cannot grow in this medium. Faust and Krieger isolated cells (called met-18b-2 cells) that acquired the ability to grow in MeLoCo, but they had not taken up any exogenous DNA, nor had they regained LDL receptors (54). The cells survived because they had acquired the ability to take up mevalonate 10–40 times more efficiently than the parental cells, and this permitted mevalonate to satisfy their bulk cholesterol requirements, even without LDL.

In 1990, we were joined by Christine Kim (now Kim-Garcia), a fiercely independent M.D./Ph.D. student who did not want to work on any of the mainline projects in our laboratory. Christine decided to see whether she could use an expression cloning strategy to identify the gene responsible for enhanced mevalonate uptake in the met-18b-2 cells. She prepared a cDNA library from the met-18b-2 cells and transfected 500 separate pools of 1000 plasmids each into dishes of human kidney 293 cells. The cells were then incubated with \(^{3}H\)mevalonate, and uptake was measured. Through multiple reiterations, Christine isolated a cDNA encoding a putative membrane protein with twelve membrane-spanning regions consistent with being a membrane transporter but having no sequence homology to any proteins that were in the databases in 1992 (55). Christine also isolated a cDNA encoding the same protein from wild-type CHO cells. When the sequences were compared, we found that the cDNA from the met-18b-2 cells harbored a single point mutation that substituted a cysteine for a phenylalanine in the tenth membrane-spanning region. We named the protein Mev. Whereas the Phe-to-Cys Mev mutant endowed cells with the ability to import mevalonate, the wild-type version did not.

We reasoned that the Phe-to-Cys mutation had altered the specificity of a membrane transporter so that it acquired the ability to transport mevalonate. The next task was to define the natural substrate for the wild-type transporter. This was no easy task. First, Christine screened many cultured cell lines to find one that did not express wild-type Mev. She identified one such line, a breast cancer line called MDA-MB-231. By transfection, she created two clones of these breast cells, one expressing wild-type Mev and the other expressing a similar amount of the mutant version. She then tested forty-two different radio-labeled compounds to see which of them was taken up more rapidly by the cells that expressed wild-type Mev but not the mutant. After many trials, Christine finally found that \(^{14}C\)pyruvate met these criteria (Fig. 3) (56). Moreover, pyruvate uptake was blocked by a series of \(\alpha\)-hydroxycinnamates that had been previously described to inhibit pyruvate uptake into red blood cells by the presumed MCT (57). We postulated that Mev was indeed the long-sought MCT, and we named it MCT1. In the met-18b-2 cells, MCT1 had undergone a point mutation that changed the specificity of the transporter from pyruvate to mevalonate.

Christine prepared an antibody against MCT1. Together with Ravindra Pathak in Richard Anderson’s laboratory, she showed that the transporter is expressed highly in erythrocytes, the basolateral epithelium of the kidney, and cardiac muscle as well as mitochondria-rich skeletal muscle fibers (56). Surprisingly, MCT1 was not expressed in liver, a finding that led Christine to use a low-stringency hybridization method to screen a hamster liver cDNA library with her MCT1 probe. The screen yielded a second MCT (which we called MCT2) that was 60% identical in its amino acid sequence to MCT1 (58). MCT2 is expressed predominantly in liver but also in mitochondria-rich skeletal muscle fibers and cardiac muscle. Like MCT1, MCT2 transports both pyruvate and lactate but has a higher affinity for both substrates compared with MCT1.

Christine’s cDNA cloning of MCT1 and MCT2 opened the floodgates. Currently, fourteen family members are known (59, 60). The range of substrates transported by different family members extends from the standard monocarboxylates, lactate and pyruvate, to aromatic
amino acids and thyroid hormones. Through expression in liver and muscle, the MCT1–MCT4 isoforms facilitate the Cori cycle, by which glucose is released from the liver and converted by glycolysis in muscle to lactate, and the lactate is returned to the liver for resynthesis into glucose. Thus, the persistence of a talented M.D./Ph.D. student can advance an important field of physiology, although never intending to do so.


We discovered the first effective treatment for congenital generalized lipodystrophy because of an accident. As summarized earlier, in 1993, we identified SREBP-1a as an activator of cholesterol synthesis mediated through sterol regulatory elements (7). At the same time, Peter Tontonoz and Bruce Spiegelman identified a related protein that they called adipocyte determination and differentiation factor-1 (ADD1) (61). They reported that ADD1 bound to an E-box in the promoter for fatty acid synthase in adipocytes, enhancing its transcription and increasing the differentiation of 3T3-L1 preadipocytes in tissue culture. Because of an artifactual premature termination codon, the ADD1 sequence did not reveal the unique membrane nature of the protein that we had found in our cDNA cloning of SREBP-1a. We subsequently determined that ADD1 was an alternatively spliced form of the SREBP-1 gene that exists in two versions, SREBP-1a and SREBP-1c, both of which have membrane domains (62). We found that SREBP-1c activates genes not only for fatty acid synthase, but for all of the other genes necessary for fatty acid and triglyceride synthesis (13). We also identified SREBP-2, a third family member encoded by a separate gene. SREBP-2 preferentially activates all of the genes necessary for cholesterol synthesis (27 enzymes) and cholesterol uptake (LDL receptor). At high levels, it also can activate the fatty acid synthesis genes. After we showed that the SREBPs must be cleaved in a regulated fashion to activate the fatty acid synthesis genes, we decided to determine the effect that the nuclear form of SREBP-1c would have on adipose tissue differentiation in mice. Accordingly, we engineered a transgene encoding the truncated nuclear form of SREBP-1c that lacks the membrane domain and thus enters the nucleus in a constitutive unregulated fashion. Transgene expression was driven by the adipose tissue-specific promoter for aP2, an adipocyte protein. This is when the accident occurred.

We expected that our transgene would cause an increase in adipocyte fatty acid synthesis and lead to enlarged and more numerous adipocytes in the transgenic mice. In fact, we observed the opposite. The mice lacked all mature adipocytes in white adipose tissue (63). Although we did not know the reason for this block in differentiation (and we still do not), we realized immediately that we had created by accident a mouse model of a human disorder, congenital generalized lipodystrophy (CGL), also known as Seip syndrome. Subjects with this disorder have a total lack of white adipose tissue. Inasmuch as they cannot store triglycerides in adipocytes, these subjects store them in the liver, which becomes massively enlarged and steatotic. CGL subjects also manifest hypertriglyceridemia and hyperglycemia caused by severe insulin resistance that elicits marked hyperinsulinemia. Their diabetes is extraordinarily difficult to control, frequently requiring injections of hundreds of units of insulin daily. Our lipodystrophic mice (63) shared all of these features with the human disease (64).

At this point, our postdoctoral fellow Ichiro Shimomura made an important observation. He found that the adipose tissue remnants in our lipodystrophic mice had a profound reduction in mRNAs for all of the adipocyte-specific genes that were tested, including leptin (63). In 1994, Jeffrey Friedman had made the remarkable discovery that adipose tissue secretes leptin, a peptide hormone that curbs appetite. Mice with genetic leptin deficiency (ob/ob mice) ingest massive amounts of food and become grossly obese, insulin-resistant, and diabetic. In one sense, our lipodystrophic mice were the opposite of ob/ob mice: they were pathologically thin instead of being enormously fat. However, the lipodystrophic mice shared all of the other characteristics of ob/ob mice, namely massive fatty liver, severe insulin resistance, and diabetes. Plasma insulin levels in our lipodystrophic mice were 60-fold above those in wild-type mice, and plasma leptin levels were barely detectable. Shimomura then asked an impertinent question: would leptin administration correct the metabolic consequences of lipodystrophy as it does for primary leptin deficiency? We had little confidence that this experiment would work, for surely adipose tissue must be essential for something other than leptin production.

Accordingly, Shimomura administered leptin to our lipodystrophic mice for twelve days through an osmotic minipump. The results were dramatic (65). Even after this short period, plasma insulin fell to normal, plasma glucose was normalized, and the fatty liver resolved completely (Fig. 4). The mice were still lipodystrophic; they had no discernable white fat, yet their insulin resistance and diabetes were reversed by administration of only a single product from adipose tissue, leptin. The hormone decreased food intake by 16% in the lipodystrophic mice,
but the metabolic resolution could not be explained solely by reduced food intake. In the absence of leptin treatment, an even greater forced reduction in food intake (30%) failed to reverse the diabetes and fatty liver (65).

Before we published these observations in Nature, we contacted our UT Southwestern colleague Abhimanyu Garg, an endocrinologist who had collected several patients with CGL. At that time, The Rockefeller University had licensed leptin to Amgen. Accordingly, in August 1999, we sent a preprint of our Nature paper to Amgen and proposed an exploratory clinical trial of leptin therapy in Garg’s patients with lipodystrophy. Six months later, in February 2000, while negotiations with Amgen were still under way, a Brief Communication appeared in Nature that threatened to abort the trial. The Communication, from Marc Reitman and co-workers in the Diabetes Branch of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), reported studies of another mouse model of lipodystrophy in which leptin administration had only a negligible ameliorating effect on blood glucose and insulin (66). They challenged our conclusion by stating, “Evidence from humans and mice supports the conclusion that leptin deficiency cannot completely explain the diabetic phenotype of generalized lipodystrophy.” They also stated that “leptin deficiency...is neither the sole cause nor the principal cause of insulin resistance in severe forms of this disease.”

The publication of the Brief Communication from the NIH cast a pall on the rationale for a clinical trial of leptin in CGL. We remained convinced by our own data in mice, and we believed that patients with CGL deserved a chance at a new treatment. Fortunately, Simeon Taylor and Phillip Gorden, two senior scientists in the Diabetes Branch, kept their minds open. They and their junior associate Elif Oral agreed to participate in the clinical trial and to enroll the CGL patients who were being followed at the NIH. Together, the Garg group and the NIH group completed the study, and the dramatic results were published in the New England Journal of Medicine in February 2002 (67). Nine female patients were treated with leptin for four months. All showed a dramatic drop in blood sugar and insulin, a dramatic reduction in liver and plasma triglycerides, and a dramatic reduction in liver size. All of the diabetic patients showed a profound reduction in the need for glucose-lowering medications. One teenage girl, who had required a massive insulin dose of 3000 units/day prior to therapy, required no glucose-lowering agent when treated with leptin. We now know that the effects of leptin persist with time. In the most recent published report, more than 100 patients with severe forms of lipodystrophy have been treated with leptin with persistent relief of diabetes, fatty liver, and hypertriglyceridemia (68).

The findings in mouse and human lipodystrophy have major implications for our notion of the role of adipose tissue in metabolism. Although adipose tissue secretes hormones in addition to leptin, including adiponectin and resistin, the absence of adipose tissue is compatible with relatively normal glucose homeostasis as long as a single hormone, leptin, is replaced.

We were intrigued with the concordant findings of severe insulin resistance with hyperglycemia, severe hypertriglyceridemia, and severe hepatic steatosis in leptin-deficient lipodystrophic and ob/ob mice. In a milder form, this constellation of abnormalities is also characteristic of humans with the metabolic syndrome and type 2 diabetes. In all of these states, the hyperglycemia is attributed to insulin resistance, primarily in liver, muscle, and adipose tissue. Insulin resistance in liver was long known to trigger an increase in hepatic gluconeogenesis due to a failure of insulin to down-regulate genes such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, which are crucial for the synthesis and secretion of glucose by the liver. On the other hand, our laboratory (69) and those of Bruce Spiegelman (70) and Pascal Ferré (71) had shown that insulin activates fatty acid synthesis in rodent liver by increasing the amount of nuclear SREBP-1c. In the insulin-resistant liver, how can insulin continue to stimulate SREBP-1c production while losing its ability to suppress gluconeogenesis?

Together with Shimomura, we focused on the paradox of simultaneous insulin resistance and sensitivity in the livers of lipodystrophic and ob/ob mice. We showed that this condition correlated with a down-regulation of IRS-2, one of the two proteins that mediates the signaling action of insulin in liver (72). We pointed out that the mixture of
insulin resistance and insulin sensitivity (which we now call “selective insulin resistance”) (73) leads to a vicious cycle: the insulin-resistance component of the cycle causes the liver to secrete excess glucose into plasma, stimulating the pancreas to produce more insulin, which fails to suppress gluconeogenesis; the insulin-sensitive component of the cycle allows the increased plasma insulin to continue to activate SREBP-1c and stimulate fatty acid synthesis, resulting in fatty liver and hypertriglyceridemia. As originally formulated by our late and lamented colleague Denis McGarry, hypertriglyceridemia causes excess fatty acids to be delivered to peripheral tissues, thereby exacerbating their insulin resistance and leading to further increases in plasma glucose and insulin and even greater production of triglycerides in liver (74). This vicious cycle characterizes not only lipodystrophy and leptin deficiency, but also the common form of type 2 diabetes associated with obesity, leptin resistance, and the metabolic syndrome.

In recent years, our laboratory has continued to study the hepatic paradox of selective insulin resistance. With postdoctoral fellow Shijie (Chris) Li, we showed that insulin induction of SREBP-1c mRNA in hepatocytes and in livers of living rats is blocked by rapamycin, the inhibitor of mammalian target of rapamycin (mTOR) (75). Rapamycin does not inhibit insulin suppression of gluconeogenesis, indicating that the two insulin signaling pathways diverge proximal to mTOR.

Currently, there is intense interest in many laboratories to identify the pathway by which insulin increases nuclear SREBP-1c mRNA and thereby increases fatty acid synthesis. If this limb of the vicious cycle can be interrupted, it is possible that some of the negative consequences of type 2 diabetes can be ameliorated. Thus, the accidental discovery of a mouse model of lipodystrophy may some day lead to effective treatments for a major epidemic that is engulfing the industrialized world.


Our foray into Niemann-Pick type C (NPC) disease began with the work of a gifted M.D./Ph.D. student, Rodney Infante. Rodney obtained his undergraduate degree in accounting. After graduation, he worked in the business world before enrolling in our medical school as a straight M.D. student. After his first year, he spent a summer working in our laboratory, and he remained for the next 5.5 years. For his thesis project, Rodney accepted the formidable challenge of purifying a membrane-bound oxysterol-binding protein.

Oxysterols such as 25-hydroxycholesterol are potent blockers of the ER-to-Golgi transport of SREBPs, and thus, they are potent suppressors of cholesterol synthesis. In fact, oxysterols are much more potent than cholesterol. The inhibitory actions of oxysterols and cholesterol are totally dependent on Insigs, polytopic membrane proteins in the ER that trap the Scap-SREBP complex and prevent its incorporation into vesicles that exit the ER (76, 77). Arun Radhakrishnan, a postdoctoral fellow, showed that cholesterol acts by binding to Scap, triggering a conformational change that causes Scap to bind to Insigs (78). However, Arun was unable to show that Scap binds oxysterols. Therefore, we postulated that ER membranes must contain an as-yet-unidentified protein that binds oxysterols and facilitates the binding of Scap to Insigs. Rodney’s task was to identify and purify this putative oxysterol-binding protein.

Rodney was a neophyte when it came to protein purification, so we encouraged him to enroll in the two-week protein purification course at Cold Spring Harbor. There he learned a great deal. It is a real technical challenge to purify a hydrophobic membrane protein that binds a hydrophobic ligand like an oxysterol. The ligand and the binding protein must both be dissolved in detergents, which create innumerable opportunities for artifacts. Nevertheless, Rodney persisted. Within a year, he had devised six chromatographic steps to obtain from rabbit liver a protein preparation that was purified by 14,000-fold. To identify the oxysterol-binding protein, he cross-linked his preparation with a derivative of 25-[3H]hydroxycholesterol. After SDS gel electrophoresis, he excised the labeled band and identified the protein by mass spectroscopy. The identified protein was encoded by the gene that is defective in a human genetic disease of cholesterol metabolism called NPC disease (79).

NPC disease is an autosomal recessive lysosomal storage disease in which cholesterol and other lipids accumulate in lysosomes, particularly in liver, spleen, lung, and brain (80). Affected individuals appear normal in infancy, but they exhibit progressive hepatic and neural degeneration, usually dying before the teenage years. NPC disease was initially lumped together with Niemann Pick type A (NPA) disease, which is caused by a deficiency of lysosomal acid sphingomyelinase. However, in the 1980s, Peter Pentchev and Roscoe Brady at the NIH realized that NPC was distinct from NPA. NPC cells had normal acid sphingomyelinase activity, yet they accumulated large amounts of cholesterol and sphingomyelin when grown in tissue culture.
Our early work had shown that cholesterol and sphingomyelin are delivered to lysosomes by the receptor-mediated uptake of LDL. After liberation from LDL, the cholesterol leaves the lysosome and is transported to the ER and plasma membrane. In 1985, Pentchev and Brady showed, indeed, that the source of excess lysosomal cholesterol in NPC cells was the LDL of the culture medium (81). When NPC cells were incubated with LDL, the LDL-derived cholesterol failed to leave the lysosomes. As a result, there was a delay in the suppression of cholesterol synthesis and the activation of cholesterol re-esterification. The gene encoding the NPC protein was isolated by positional cloning in 1997 (82). Its sequence predicted a protein of 1278 amino acids with thirteen transmembrane helices. It was named NPC1. The protein was localized to lysosomal membranes but had never been purified, and there was no information about its biochemical properties. Peter Lobel and colleagues showed that a minority of NPC patients owe their disease to defects in another protein that they named NPC2 (83). NPC2 (then called HE1) had been identified earlier as a cholesterol-binding protein in epididymal fluid. Lobel showed that NPC2, a 132-amino acid soluble protein, is also targeted to lysosomes, where it resides in the lumenal fluid. The protein was missing in patients with NPC disease who did not harbor a mutation in NPC1. Defects in NPC1 or NPC2 produce the same clinical phenotype, suggesting that they may act sequentially in transporting cholesterol out of lysosomes (84, 85).

Rodney’s discovery of NPC1 as an oxysterol-binding protein intrigued us. We were even more enthralled when he showed that the protein also binds cholesterol (79). By this time, Radhakrishnan had shown that the oxysterol sensor for SREBP processing is not an unknown protein; indeed, it is Insig itself, which binds oxysterols but not cholesterol (86). Rodney’s NPC1 protein was not involved in feedback regulation of SREBP processing, but it was even more interesting because it was crucial for cholesterol export from lysosomes, a process that was totally mysterious.

Rodney then expressed deletion mutants of NPC1 so as to identify the domain that bound sterols. Here, we got another surprise. Among the thirteen transmembrane helices of NPC1, five show sequence resemblance to membrane-spanning sequences in Scap, HMG-CoA reductase, and certain other proteins that are regulated by sterols. We had noticed this ~170-amino acid domain originally in Scap, naming it the sterol-sensing domain (87). We expected that sterols would bind to this domain of NPC1. However, Rodney showed that sterols do not bind to the membrane domain of NPC1, at least under the conditions of his in vitro assay. Rather, sterols bind to the NH2-terminal end of NPC1, which is a sequence of 240 amino acids that projects into the lysosome lumen. When expressed as a truncated protein without the membrane domain, the NH2-terminal domain (NTD) is secreted into the culture medium. After purification, it binds cholesterol and 25-hydroxycholesterol with saturation kinetics (79). Cross-competition studies with related sterols indicated that the binding site recognizes the sterol nucleus, including the 3β-hydroxyl on the A-ring, but it is indifferent to hydrophilic substitutions on the isoctyl side chain on the D-ring (88). The protein does not recognize epicholesterol, which differs from cholesterol only in the orientation of the 3-hydroxyl group, which is in the α-position in epicholesterol. Rodney also prepared recombinant NPC2, and he found that the soluble protein recognized the opposite end of the sterol (88). NPC2 bound epicholesterol, but it did not bind 25-hydroxycholesterol, suggesting that it recognizes the hydrophobic isoctyl side chain on the D-ring, but not the 3β-hydroxyl on the A-ring.

At the same time that Rodney was performing his comparative binding studies, Lobel and Ann Stock published the structure of NPC2 bound to cholesteryl sulfate as determined by x-ray crystallography (89). Their structure supported the idea that NPC2 binds cholesterol by recognizing the sterol rings and the isoctyl side chain, but the 3β-hydroxyl does not interact with the protein. Indeed, NPC2 bound cholesteryl sulfate even with the bulky sulfate attached to the 3β-hydroxyl. Rodney showed that cholesteryl sulfate does not bind to NPC1, further heightening the differences between NPC1 and NPC2 (88).

Mouse knock-in experiments confirmed that the cholesterol-binding activity of NPC1(NTD) is essential for NPC function in living animals. In preparation for these studies, Rodney and another M.D./Ph.D. student, Michael Wang, performed alanine scanning mutagenesis of NPC1(NTD) to identify residues that were crucial for sterol binding. They identified two adjacent amino acids (Pro-202 and Phe-203) that, when changed together to alanine, eliminated cholesterol binding in vitro. Another graduate student, Lina Abi-Mosleh, showed that full-length NPC1 containing these adjacent alanine substitutions failed to complement the defect in NPC1-deficient CHO cells (90). Building upon these in vitro observations, our colleague Guosheng Liang, together with postdoctoral fellow Xuefen Xie, produced knock-in mice homozygous for the P202A/F203A mutation in the npc1 gene and showed that they exhibit a clinical and pathologic syndrome indistinguishable from that in npc1<sup>−/−</sup> mice, the classic model for NPC1 deficiency (91).
Rodney’s biochemical observations that NPC2 and NPC1 were both cholesterol-binding proteins suggested that the two proteins might transfer cholesterol between themselves (92). In his initial studies, Rodney had observed that cholesterol bound very slowly to NPC1(NTD) at 4 °C. Moreover, after binding cholesterol, NPC1(NTD) slowly transferred the bound sterol to phospholipid liposomes. However, when NPC2 was added, NPC1(NTD) bound cholesterol many times more rapidly, and it also transferred cholesterol much more rapidly to liposomes. A naturally occurring NPC2 mutant that does not bind cholesterol failed to accelerate association and dissociation of cholesterol from NPC1(NTD). We hypothesized that NPC2 acts as a cholesterol shuttle, either delivering cholesterol to NPC1 or removing it. To test this hypothesis, Rodney designed a direct transfer assay in which he first isolated NPC2 with [3H]cholesterol bound and then incubated this complex with NPC1(NTD). Whereas the NTD bound free cholesterol extremely slowly, binding was accelerated by orders of magnitude when the sterol was delivered directly by NPC2 (92).

At this point, our studies were advanced immensely through a collaboration with Hyock Kwon, a postdoctoral fellow in the laboratory of our colleague Johann Deisenhofer. Together, Hyock and Rodney crystallized the NTD of NPC1 with and without bound cholesterol (93). The structures confirmed the conclusions drawn from the binding studies. Cholesterol was buried in a deep pocket within the NTD, which formed tight contacts with the sterol ring and particularly with the 3β-hydroxyl group. The isooctyl side chain extended through a small tunnel to the surface, projecting away from the binding pocket. Importantly, the entry to the cholesterol-binding pocket was occluded by three α-helices that must have moved aside to admit the cholesterol and then closed behind it to lock the sterol in place.

Rodney and Michael Wang performed additional alanine scanning mutagenesis of NPC1(NTD), and they identified a small patch of residues on the surface of the NTD that were not required for the NTD to slowly bind free cholesterol but were essential for rapid acceptance of cholesterol transferred from NPC2 (93). We called these “transfer” mutants, distinct from the “binding” mutants. Later, Michael and Rodney made a similar alanine scanning mutagenesis study of NPC2 and identified a similar patch of residues on the surface of NPC2 that were required to transfer cholesterol to NPC1(NTD) (94). Working together with Hyock and using the crystal structure published by Lobel and Stock, Michael and Rodney were able to align the two proteins such that the transfer patches on both proteins met each other, and the bound cholesterol was in the same plane in the two proteins (94). On the basis of this alignment, we proposed a model that we called the hydrophobic handoff (Fig. 5).

The hydrophobic handoff model (93, 94) postulates that soluble NPC2 binds cholesterol immediately after acid lipase releases it from the cholesteryl esters of LDL. NPC2 carries the cholesterol to the lysosomal membrane, where it binds to the NTD of NPC1, which projects through the glyocalyx, the thick carbohydrate lining that prevents lysosomal proteins from direct contact with the limiting membrane (95). Binding of NPC2 induces a conformational change in the NTD of NPC1 that moves aside the obstructing helices and opens the sterol-binding pocket. Once this entrance is opened, cholesterol slides directly from NPC2 to NPC1. After the handoff, the NPC2 dissociates, and the surface helices on the NTD close again, locking the cholesterol in the binding pocket. The great virtue of this handoff is that it shields the cholesterol from water. From the moment that cholesterol leaves LDL, it is always bound to a protein, and it never has the opportunity to crystallize, which would be a catastrophic event in the lysosome. This model has been expanded recently by Suzanne Pfeffer and her colleague, who have shown that NPC2 binds to a lumenal loop of NPC1 that is adjacent to the NTD (96). This binding may facilitate the transfer of cholesterol from NPC2 to the NTD of NPC1.

If the hydrophobic handoff model turns out to be correct, it still leaves a major problem: how does the cholesterol move from the NTD of NPC1 to enter the membrane and emerge on the other side for transport to the ER and plasma membrane? Here, it is likely that the thirteen membrane-spanning helices of NPC1 play a role, but how they function is a complete mystery. In fact, we know very little about how cholesterol is transported between any...
organelles in a cell. This may be a topic for a future excursion from the beaten path.


We were attracted to ghrelin as soon as we found out that it is the only peptide hormone that bears a covalently attached fatty acid. Even more unique is its distinction as the only known eukaryotic protein that bears an eight-carbon fatty acid (octanoate). Lipid modifications of proteins have fascinated us ever since our studies of protein farnesylation, and we were eager to uncover the enzyme that attaches octanoate.

Ghrelin was discovered in 1999 by Masayasu Kojima, Kenji Kangawa, and associates in Osaka as a stomach-derived hormone that releases growth hormone from pituitary cells (97). They characterized ghrelin as a 28-amino acid peptide with an octanoate attached in ester linkage to a serine at the third position from the NH₂ terminus. Ghrelin’s activity in releasing growth hormone from the pituitary and hypothalamus is strictly dependent on the octanoate moiety.

Ghrelin came to prominence when its plasma concentration was shown to rise dramatically before meals and to decline immediately after eating in humans and rodents (98, 99). Moreover, injection of excess ghrelin increased food intake in humans as well as rodents (100 –102). These findings led to the hypothesis that ghrelin is responsible for hunger before meals and satiation after eating. The validity of the hunger hypothesis came into question when the gene for ghrelin or its receptor was eliminated in mice through germ line recombination (103–105). These defective animals grew normally and showed only inconsistent and mild decreases in food intake and body weight.

Frankly, we were very skeptical. Having failed with the first presumed ghrelin O-acyltransferase was not a member of the MBOAT family at all.

Jing cloned cDNA copies of fifteen MBOATs by PCR of mRNAs from mouse stomach. To assay these putative enzymes for ghrelin O-acyltransferase activity in intact cells, Jing first identified an insulin-secreting rat cell line called INS-1, which is capable of cleaving transfected preproghrelin to ghrelin but is not capable of acylating the ghrelin (107). He then established a reverse-phase chromatographic assay to separate acylated ghrelin from desacyl ghrelin. He transected the INS-1 cells with a plasmid encoding preproghrelin plus a plasmid encoding one of the fifteen MBOATs. He extracted the ghrelin from the cells and used his reverse-phase column to determine whether it was acylated. All results were negative. At this point, we had no assurance that the INS-1 cells could acylate ghrelin even if the enzyme was supplied. Perhaps the enzyme required the assistance of another protein that was missing from the cells. It was also possible that the presumed ghrelin O-acyltransferase was not a member of the MBOAT family at all.

Jing pointed out that he had been unable to amplify one of the sixteen MBOATs from the mouse stomach mRNA. He proposed to make new attempts to isolate this last one. He used his reverse-phase column to determine whether the sixteenth MBOATs was acylated ghrelin even if the enzyme was supplied. Perhaps the enzyme required the assistance of another protein that was missing from the cells. It was also possible that the presumed ghrelin O-acyltransferase was not a member of the MBOAT family at all.

Jing pointed out that he had been unable to amplify one of the sixteen MBOATs from the mouse stomach mRNA. He proposed to make new attempts to isolate this last one. Frankly, we were very skeptical. Having failed with the first fifteen MBOATs, what was the probability that the sixteenth would succeed? Nevertheless, Jing persisted. He found that the problem was that the mouse genome project contained an incorrect annotation of the exon at the 5‘-end of the gene, so Jing’s primers could not have worked. To circumvent this problem, Jing pieced together the putative cDNA by ligating synthetic oligonucleotides that corresponded to the available partial mouse cDNA sequence. He used 5‘-rapid amplification of cDNA ends and nested PCRs to extend his cDNA to the 5‘-end of the mRNA (107). This was an enormous amount of work simply to test whether the sixteenth MBOAT would be the sought-after one. Almost miraculously, these experiments worked. When transfected into INS-1 cells, Jing’s synthetic sixteenth MBOAT cDNA produced octanoylated ghrelin, as did the authentic cDNA that he subsequently cloned. After our paper had been reviewed and accepted for publication in Cell, Gutierrez et al. (108) from Eli Lilly & Company submitted a paper to the Proceedings of the National Academy of Sciences of the United States of America in which they identified the same MBOAT that we had described as a mediator of ghrelin octanoylation.
They studied a line of thyroid carcinoma cells that secreted octanoylated ghrelin, and they screened siRNAs targeting candidate MBOATs for their ability to block ghrelin acylation.

The sixteenth MBOAT in Jing’s studies and the one identified by Gutierrez et al. corresponded to MBOAT-4 in Hofmann’s original list. We renamed this enzyme GOAT, which stands for ghrelin O-acyltransferase (107). The tissue distribution of GOAT mRNA coincided with the distribution of ghrelin, being found largely in the stomach and upper small intestine. We established an in vitro assay to measure the transfer of octanoate from octanoyl-CoA to proghrelin (109). Ghrelin corresponds to the first twenty-eight amino acids of proghrelin, which is generated after the protein is inserted into ER membranes and the signal sequence has been cleaved. After transport to the Golgi, proghrelin is cleaved by a furin-like protease to generate the mature 28-amino acid ghrelin. GOAT attaches octanoate to Ser-3 of proghrelin, which corresponds to the site originally identified in ghrelin by Kojima and Kangawa. We found that GOAT did not require a full-length protein for activity. Indeed, the enzyme transferred octanoate to a peptide corresponding to the first five amino acids of proghrelin. GOAT was sensitive to end product inhibition by octanoylated peptides. The most potent inhibitor (Ki = 0.2 μM) was a pentapeptide in which diaminopropionic acid was substituted for Ser-3, and the octanoate was attached to this residue in amide linkage rather than the less stable ester bond (107). These studies raised the possibility of designing inhibitory peptidomimetics to inhibit GOAT in vivo provided a relevant clinical indication could be identified.

In an effort to understand the true physiologic or pathologic role of ghrelin and to confirm the role of GOAT in producing the hormone, we collaborated with scientists at Regeneron Pharmaceuticals, Inc., to eliminate the Goat gene in mice by germ line recombination. In Dallas, these experiments were conducted by Tong-Jin Zhao, a skilled postdoctoral fellow from Tsinghua University, with expert help from our colleague Guosheng Liang. Because there is some ambiguity in nomenclature, in the following discussion, we use the term “ghrelin” to denote the octanoylated 28-amino acid mature form of the hormone that circulates in blood. “Desacyl ghrelin” denotes the uncylated form, which is also found in the circulation. GOAT-deficient mice had no circulating ghrelin, confirming the indispensable role of GOAT in mediating the acylation reaction (110). Circulating levels of desacyl ghrelin were actually elevated in the GOAT knock-out mice. The GOAT-deficient mice grew normally and consumed normal amounts of food. Moreover, when fasted, they showed the same signs of hunger as did wild-type mice. We were not surprised by these results because they are the same as observed in knock-out mice lacking ghrelin or the ghrelin receptor.

Persisting in the idea that ghrelin must play a role in appetite regulation, we decided to determine whether wild-type mice would outlive Goat+/− mice when placed in conditions in which food was limited. We planned to place one wild-type mouse and one Goat−/− mouse in the same cage but provide an amount of food that was sufficient only for one. However, prior to performing this experiment, we decided to see what would happen if we kept the wild-type mouse and the Goat−/− mouse in separate cages but provided each with only 40% of the amount of food that they normally consumed in one day. We called this “60% calorie restriction,” and the results were dramatic. Faced with 60% calorie restriction, wild-type and Goat−/− mice became progressively hungrier. The mice were fed each day at 6 p.m. After a few days, all of the mice, Goat−/− as well as wild-type, consumed their entire food allotment within 1 h. Over the first four days, wild-type and Goat−/− mice lost 30% of their body weight and more than 80% of their body fat. We measured blood sugar levels at 5:30 p.m., 23 h after the mice had completed their last meal. Despite their fat loss, wild-type mice were able to maintain blood sugar levels in the viable range, always above 40 mg/dl. In dramatic contrast, by day 7, Goat−/− mice developed severe hypoglycemia with blood sugar levels reaching as low as 12 mg/dl (Fig. 6). At this point, the Goat−/− mice appeared moribund, and they were killed (110).

We also measured plasma hormone levels at 5:30 p.m. Wild-type mice showed progressive increases in ghrelin, desacyl ghrelin, and growth hormone. By day 7,
the two hormone levels were more than 4-fold above those in ad libitum fed wild-type mice. In Goat\(^{-/-}\) mice, ghrelin was undetectable, desacyl ghrelin rose to wild-type levels, and growth hormone barely increased. Hypoglycemia in Goat\(^{-/-}\) mice was prevented when the animals received constant infusions of either ghrelin or growth hormone via an osmotic minipump beginning three days prior to instituting calorie restriction (110).

In a subsequent study, Tong-Jin, together with M.D./Ph.D. student Robert Li and postdoctoral fellow Daniel Sherbet, studied the diurnal pattern of blood sugar in the calorie-restricted mice (111). Even after seven days, the Goat\(^{-/-}\) and wild-type mice both had normal blood sugar levels of 60 mg/dl when measured at 9 a.m., 15 h after finishing their last meal. Thereafter, wild-type mice maintained stable blood sugar levels in this range throughout the day of fasting. In Goat\(^{-/-}\) mice, by 2 p.m., the blood sugar began to decline significantly, and it reached its nadir at 5:30 p.m., 23 h after the last meal. In both strains, glucose rose immediately after eating the next meal. The same end-of-day hypoglycemia was observed in calorie-restricted ghrelin knock-out mice, indicating that hypoglycemia in the Goat\(^{-/-}\) mice was indeed caused by ghrelin deficiency. The above studies indicated that this hypoglycemia required two events: 1) chronic calorie restriction to deplete body fat stores nearly completely and 2) a prolonged fast of 23 h in the fat-depleted state.

In their most recent experiments, Tong-Jin and Sherbet infused \(^{3}\)H\)glucose into wild-type and Goat\(^{-/-}\) mice and demonstrated that the end-of-day decrease in blood sugar is caused by a 60% reduction in glucose production (111). The decreased glucose production could not be attributed to a decrease in the production of gluconeogenic enzymes at the mRNA level. Indeed, the Goat\(^{-/-}\) mice had nearly undetectable levels of plasma insulin and markedly elevated levels of glucagon, as would be expected in the hypoglycemic state. Moreover, the liver responded to the glucagon by generating marked elevations in mRNAs encoding phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, two glucagon-induced enzymes of glucose production (110, 111). Plasma levels of lactate and pyruvate, two important substrates for gluconeogenesis, were reduced by 50% in the hypoglycemic Goat\(^{-/-}\) mice. Moreover, the hypoglycemia could be reversed by injections of lactate, pyruvate, or alanine, all of which can be converted to glucose. Hypoglycemia was also corrected by infusing octanoate, a medium-chain fatty acid that cannot be converted to glucose but can provide energy and reducing equivalents that are necessary for gluconeogenesis (111). Thus, hypoglycemia in calorie-restricted mice appears to be due primarily to a limitation in available substrates and energy rather than to a problem with the production of gluconeogenic enzymes. A crucial question is how ghrelin-induced growth hormone can maintain the levels of gluconeogenic substrates under a condition in which there are no fat stores and where there is prolonged food deprivation. Solving this problem will shed light on a hitherto unknown function of growth hormone as well as ghrelin.

Starvation is a frequent bottleneck in the evolution of animals, and nature clearly has selected for mechanisms to maintain blood sugar at levels sufficient to keep the heart beating and the brain functioning. Our evidence indicates that ghrelin is an essential agent in blood sugar maintenance. Depleted fat stores and food deprivation are observed in humans suffering from chronic starvation or from anorexia nervosa. In both cases, plasma levels of ghrelin and growth hormone are elevated, as they are in calorie-depleted fasted mice (112). We therefore believe that ghrelin helps to maintain blood sugar by stimulating growth hormone secretion in humans. Our excursion into GOAT and ghrelin began with the persistence and skill of Jing Yang and was extended by the meticulous animal experiments initiated by Tong-Jin Zhao and Guosheng Liang.

Concluding Remarks

The six excursions described in this Reflections article were made possible by three extraordinary privileges that we have enjoyed over four decades. The first is a steady parade of talented students and postdoctoral fellows from around the world who came to a scientifically youthful place like Dallas, Texas, braved difficult challenges, and solved problems that often seemed unsolvable. In this Reflections article, we cite the research of students and postdoctoral fellows from ten different countries in addition to the United States. Moreover, our continuing work on cholesterol metabolism has been aided by students and postdoctoral fellows from seventeen additional countries (a total of twenty-seven countries). We sincerely hope that the United States continues to be a magnet for the brightest talent from around the world.

The second privilege is generous and sustained philanthropic support from enlightened citizens of Dallas who believed in us and our work. We particularly thank the late Erik Jonsson, Ross Perot, and Peter O’Donnell, Jr., three giants of Dallas. Each of them made an enormous difference not only to us, but to our medical school and to our city. When we embarked on each of our six excursions, we had no preliminary data of the type required by review.
committees of the NIH. No committee would have funded experiments that were supported only by the following outrageous hypotheses.

1) Macrophages have receptors that scavenge a variety of abnormal macromolecules, one of which is responsible for cholesterol accumulation in atherosclerotic plaques. 2) Blindness in young boys with choroideremia is caused by a failure to attach geranylgeranyl groups to Rab proteins. 3) Studies of mutant CHO cells that take up mevalonate might lead to the molecular identification of the long-sought MCT. 4) Studies of an artificial model of lipodystrophy in mice might lead to the first effective treatment for the disease in humans and provide fundamental insights into selective insulin resistance in livers of humans with type 2 diabetes. 5) The polytopic membrane protein NPC1 contains a cholesterol-binding site, not in its membrane domain but in its soluble NH2-terminal extension that accepts cholesterol from NPC2, thus providing a mechanistic explanation for a devastating genetic disease. 6) The essential function of ghrelin is to maintain blood sugar during chronic starvation.

In each case, we used our philanthropic underpinning to initiate these high-risk studies, none of which would have been possible without such support. In contrast, we used our generously funded long-term NIH program project grant to support our work on the regulation of cholesterol metabolism, which over the years became mainstream and thus approvable by NIH review panels.

The third privilege that we have enjoyed is working with brilliant colleagues at the University of Texas Southwestern Medical School in Dallas. Under the inspired leadership of our former president, Kern Wildenthal, our school recruited many of the finest scientists whose work spans the spectrum of modern biomedical research. With some, we have engaged in formal collaborations. With others, we have benefitted from endless hours of excited discussions on all manner of topics. Their insights shine through our work.

Fig. 7 documents the inevitable changes that have occurred over our forty-six years of friendship and partnership.

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