Oral Delivery of Protein Drugs: Driver for Personalized Medicine?

Günter Müller*

Ludwig-Maximilians-University Munich, Biocenter, Department Biology I, Genetics, 82152 Martinsried near Munich, Germany
Present address: Sanofi-Aventis Deutschland GmbH, R and D Diabetes, Industrial Park Höchst, 65926 Frankfurt am Main, Germany

Abstract
The pathogenesis of common diseases, such as metabolic diseases, is caused by the complex and individual interplay of many susceptibility genes, which necessitates both personalized diagnosis and therapy. Small-molecule drugs which adequately address the multiple tissue-specific target proteins affected probably will not become available in near future. In contrast, therapeutic proteins, such as growth factors and antibodies, specifically replacing or inactivating the corresponding susceptibility gene products, are currently being identified with increasing efficacy. However, the failure to be administered by the oral route and to reach the cytoplasm of the diseased cells typically prevents their therapeutic use. Recent developments suggest that these limitations may be overcome by encapsulation of therapeutic proteins into nanoparticles or their covalent modification with glycolipid (glycosylphosphatidylinositol, GPI) structures. These act as membrane anchors for so-called GPI-anchored proteins and direct certain attached passenger proteins from lipid raft areas of the plasma membrane *via* cytoplasmic lipid droplets into small vesicles. These leave the donor cells and transfer the GPI-anchored proteins into the cytoplasm of acceptor cells. This pathway may enable the transport of therapeutic proteins across the intestinal barrier into the circulation and eventually across the plasma membrane of the diseased target cells. For therapy, a number of challenges remains to be tackled, in particular, control of release from the GPI anchor which determines the pharmacokinetic and pharmacodynamic profiles. Together these findings nourish the hope that oral path finding to drug targets by encapsulation and covalent modification of therapeutic proteins may enable personalized therapy of common diseases.

Personalized therapy

Introduction

Common, multifactorial and chronic diseases typical for the Western life style are characterized by high and still increasing incidence, meanwhile also in developing countries, complex etiology, long-lasting pathogenesis and extremely variable phenotypes between the affected patients. They are caused by individual and complex interactions between multiple susceptibility genes and environmental factors (Gibson, 2009). Consequently their cure requires personalized diagnosis and polytherapy (Janssens et al., 2004; Müller, 2010). However, one should always keep in mind that the appropriate drugs have not only to be applied to the right patient, but have also to be transported to the right localizations within the patient's body *via* acceptable or accepted routes (Rajendran et al., 2010).

Email: Guenter.Mueller@sanofi-aventis.com

Type II diabetes

A highly representative example for a complex common disease with 5 to 10 % frequency in the Western countries represents type II diabetes (Müller, 2010). Permanently elevated blood glucose in fastened and postprandial states leads to the non-enzymic glycation of long-living intra- and extracellular proteins accompanied by functional impairments in multiple cells and tissues. Over years this sequence of events causes the chronic diabetic late complications which affect many different organ systems, such as eyes, neurons, kidney, legs, blood vessels and heart with varying degrees and huge inter-interindividual variability in the often severe clinical outcomes, including reduced life quality and expectancy.

Many distinct organ systems are afflicted in type II diabetes, such as pancreatic ß-cells, the brain, the intestine, adipose, muscle and liver cells. They control *via* complex networks insulin secretion, glucose sensing, insulin action and energy intake, which in concert regulate glucose and lipid metabolism and guarantee their homeostasis (Lindgren, 2007; Prokopenko et al., 2008). In consequence, gross mutations and single nucleotide polymorphisms in the majority of these so-called susceptibility genes may compromise the pleiotropic and interconnected signalling pathways and thereby contribute to the pathogenesis of type II diabetes as is the case for other common complex diseases (Ridderstrale and Groop, 2009). For type II diabetes the number of susceptibility genes has been estimated to account for 2000 to 4000 (Florez, 2008). Importantly, their individual contribution to the increase in the relative disease risk is typically rather small, ranging from 0.1 to 5 % (Müller, 2010). Thus only combinations of considerable numbers of different susceptibility genes superimposed with various environmental factors ultimately trigger the pathogenesis. It is the huge number of these different putative combinations which creates the enormous variability of the phenotypes and clinical outcomes between patients affected by the same complex common disease and necessitates their personalized diagnosis and therapy (Lusis et al., 2008). However, as we all know, personalized medicine has remained fiction or vision so far, best exemplified by the current therapy of type II diabetes with just six different approved drug classes. What are the reasons for this failure, at least with regard to the treatment options?

Current problems with drug discovery

The past two decades of drug discovery have been characterized by very low numbers of approved and really innovative novel small-molecule drugs despite enormous

technical and financial efforts required for high-throughput technologies and structural knowledge about the target proteins (Billingsley, 2008). Apparently, small-molecule drugs, which typically can be administered orally and easily penetrate into cells, are very difficult to discover, in part due to inherent, i.e. size-based, problems with their selectivity and safety. Gene therapy and regenerative medicine seem to be very promising approaches, but in the long term only, and important issues remain to be solved, such as introduction of safe vector systems and exclusion of proliferative potential (Li and Huang, 2007). Moreover, both approaches seem unlikely to be applicable for the simultaneous treatment of different target cells, as is required for common complex diseases. Therapeutic proteins benefit from rapid identification and validation in parallel with the discovery of novel disease susceptibility genes and from their very selective and safe action. However, both approaches suffer from their inherent resistance toward oral application and penetration into the diseased target cells. Nevertheless, therapeutic proteins and antibodies, in principle, are capable of substituting for and inhibiting, respectively, each potential susceptibility gene product, being it a soluble component of the serum, such as hormones, growth factors and enzymes, or a cell surface receptor, or an intracellular signalling or structural protein or enzyme. Taken together, therapeutic proteins have the potential for personalized polytherapy provided strategies for their oral administration and cellular penetration can be realized in the near future.

Oral drug delivery

So far the long path from intake through the mouth to the drug target within the body across the intestinal and cellular barriers has remained the major hurdle for the application of protein therapeutics in humans. The intestine is lined by a monolayer of epithelial cells, so-called enterocytes, which are surrounded by a bipartite plasma membrane. The apical plasma membrane area is directed toward the intestinal lumen and forms microvilli, the basolateral area is directed toward the blood vessels and faces underlying tissues (Figure 1). The enterocytes are interconnected to each other by specific protein complexes, so-called tight junctions, which seal the monolayer in a rigid fashion. Any nutrient, chemical or drug taken up orally has two options for passage from the intestinal lumen into the circulation and the underlying tissues. It can either use the paracellular route thereby crossing the tight junctions or the transcellular route thereby consecutively crossing the apical plasma membrane, cytoplasm and basolateral plasma membrane. Small-molecule drugs, molecular weight less than 500 Da, can engage both pathways. In contrast, macromolecules, such as proteins and nucleic acids, fail to use either pathway. In fact, the so-called oral bioavailability of even very small proteins, such as insulin, molecular weight about 5000 Da, typically accounts for less than 1 %. During the past 60 years biotechnologists and pharmacologists have undertaken enormous efforts to overcome these intestinal and cellular barriers for the application of putative protein drugs in humans (for reviews see Uhrich et al., 1999; Yokoyama, 2005; Ebbesen and Jensen, 2006; Kirpotin et al., 2006; Yang et al., 2007; Matsumara, 2008; Soussan et al., 2009; Gullotti and Yeo, 2009; Mok et al., 2009). In the following two technologies are presented which hopefully will fulfil the dream of needle-free admininistration of protein drugs, i.e their encapsulation into microspheres or nanoparticles, which take the paracellular route, and their covalent modification with the so-called GPI anchor, which acts as signal for transcellular transport (Figure 1).

For *in vitro* testing of and discrimination between transcellular and paracellular transport of proteins a cell culture-based so-called multiplex assay system was developed (Figure 2). For this, cultured enterocytes (e.g. CaCo-2) are grown in sealed monolayers with correctly formed tight junctions and adherent to a filter plate, which enables exchange of small molecule nutrients and ions, only. This assembly separates the apical area above the filter plate from the basolateral area below the filter plate. The protein drug candidates to be tested are continuously applied to the apical area and their transport across the cell layer is continuously monitored, either simply by conventional analytics of the incubation medium in the basolateral area or in case of using fluorescently labelled proteins by confocal laser scanning microscopy and subsequent virtual reconstruction of 3-dimensional pictures from the original 2-dimensional images. Thereby the transcellular passage of a fluorescent protein in the X-Z direction from the apical surface into the depth of the monolayer can be visualized directly in real time (Figure 2, lower inset). Simultaneously the electrical resistance between the apical and basolateral areas across the monolayer is measured which represents a parameter for the transcellular *vs*. the paracellular route (see below). In addition, putative effects of the protein drug during transcellular transport on glucose/lipid metabolism as well as protein/DNA synthesis can be detected in parallel. For this, scintillation cocktail is incorporated in the filter plate which monitors accumulation of radiolabelled glycogen, lipid, protein and DNA formed in the adherent monolayer enterocytes from corresponding radiolabelled building blocks (Figure 2). This multiplex assays system has meanwhile been adapted to an automated low-throughput scale.

Paracellular transport of protein drugs by encapsulation

As a representative example for paracellular transport of protein drugs by encapsulation, nanoparticles assemblied from the biomaterials chitosan, alginate, mannans and γ-polyglutamic acid at specific ratios are briefly discussed (Mathiowitz et al., 1997; Lin et al., 2007; Mathiowitz, 2008). Along their paracellular route these nanoparticles are faced with several challenges. First they have to adhere to the negatively charged mucus and glycocalyx layers of the small intestine (see Figure 1), which is achieved by their positive net surface charge. Following infiltration of these layers the nanoparticles have to trigger opening of the tight junctions, presumably by causing redistribution of some of their constituent proteins, e.g. F-actin and ZO-1, for their subsequent passage between two neighbouring enterocytes. However, immediately thereafter the tight junctions have to reseal in order to maintain the intestinal permeability barrier. Otherwise the uncontrolled flux of nutrients and infectious components from the intestinal lumen into the circulation could lead to a catastrophy.

The opening and closure of the tight junctions during paracellular transport can be followed by measurement of the electrical resistance with the multiplex assay system introduced above (see Figure 2). Immediately following addition of correctly assembled nanoparticles the electrical

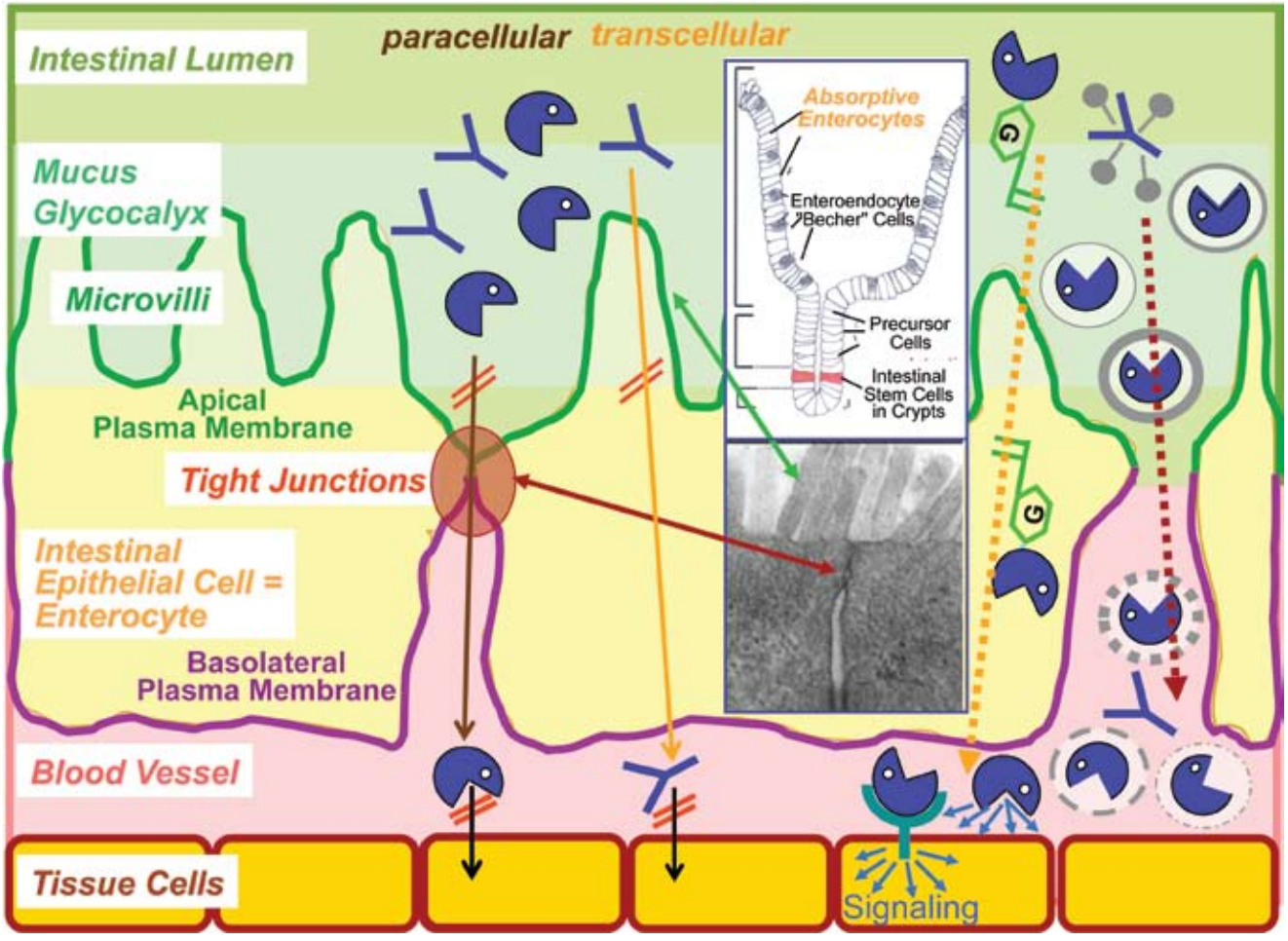

Figure 1. The long path to pharmacological drug targets – Transport of drugs across the intestinal and cellular barriers. Therapeutic proteins and antibodies can not use the paracellular or transcellular route for passage from the intestinal lumen into the circulation and underlying tissue target cells. Currently two main strategies are being evaluated to overcome the intestinal barrier formed by the mucus and glycocalyx and then either the apical plasma membrane of the microvillli, the cytoplasm and the basolateral plasma membrane of the enterocytes or the tight junctions and subsequently the plasma membrane of the target tissue cells: Transient opening of the tight junctions by permeation enhancers, i.e. detergent-like molecules, or nanoparticles with the protein drug incorporated and, alternatively, transport across the enterocytes by covalent modification of the protein drug, which acts as signal for transcellular transport. The type of the nanoparticle materials (indicated by different thickness) may determine the tissue distribution of the protein drug.

resistance dramatically drops (Mathiowitz et al., 1997). After their removal by several washing cycles of the filter plate the electrical resistance does not decline further and instead gradually increases with time to about the initial values within the next 30 to 60 min. This procedure can be repeated several times with similar results showing the transient and reversible nature of the opening and closure of tight junctions by these nanoparticles. Finally, after arrival of the nanoparticles at the blood stream they have to release their protein load, which is achieved by their dissociation. The assembly of this type of nanoparticles is strongly dependent on the pH. They are stable at pH 6.6 as adjusted during their production and dissociate at pH 7.4 as prevalent in the blood. Here the released protein drugs may exert their physiological action or eventually they have to be further transported across the plasma membrane into the cytoplasm of the target cells (Mathiowitz et al., 1997). Importantly, there are many critical requirements for nanoparticles for (chronic) use in humans, predominantly concerning safety, non-immunogenicity and non-accumulation in the body due to their proper degradation and excretion. So far, materials fulfilling all these criteria are not available and consequently there is urgent need for the discovery and development of novel biomaterials.

Transcellular transport of protein drugs by covalent modification

As a representative example for transcellular transport of protein drugs by covalent modification the concept of glycosylphosphatidylinositol-anchored proteins, abbreviated GPI-anchored proteins in the following is introduced here. In contrast to typical transmembrane proteins, GPI-anchored proteins are embedded in the outer leaflet of the plasma membrane by a specific GPI glycolipid which consists

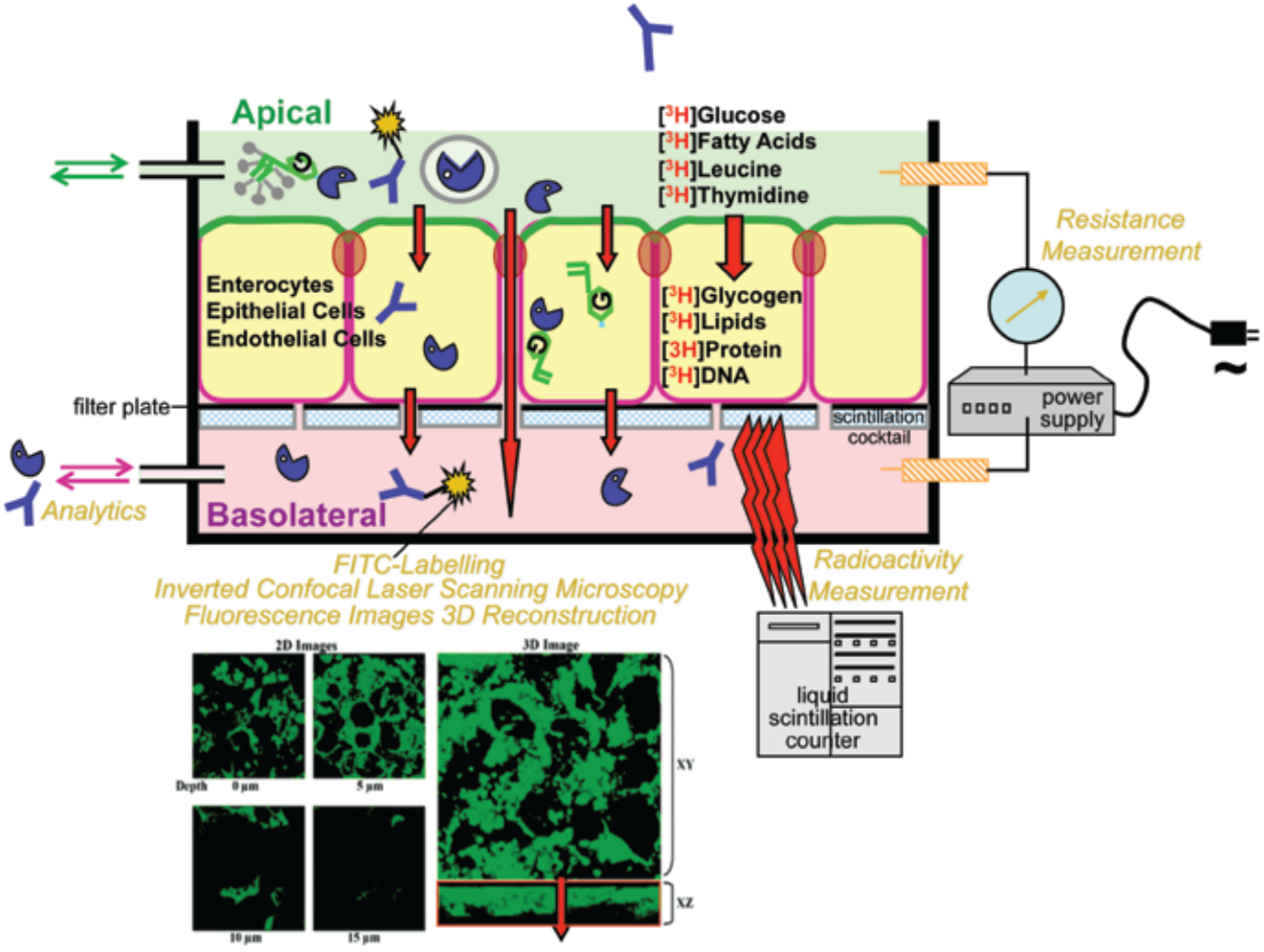

Figure 2. Multiplex assay system for simultaneous measurement of transcellular vs. paracellular transport and physiological activity of protein therapeutics. Transport of unlabelled or fluorescently labelled free, covalently modified or encapsulated therapeutic proteins and antibodies is assessed by their application to the apical area of the enterocyte monolayers and determination of their concentration (e.g. by ELISA) or confocal laser scanning microscopy. The reconstruction of a 3D image demonstrating the transport of a fluorescently labelled protein across the endothelial monolayer (X-Z direction) is shown in the inset below. Simultaneous measurement of the electrical resistance across the monolayer and of the amount of radiolabelled macromolecules synthesized from the corresponding radiolabelled building blocks and accumulated in the enterocytes adherent to the filter plate provides information about para- *vs*. transcellular transport mode and about putative effects of the protein drug on macromolecule synthesis during transport.

of phosphatidylinositol followed by a glycan core and a phosphodiester-ethanolamine bridge (Brewis et al., 1995; Nosjean et al., 1997; Ikezawa, 2002; Orlean and Menon, 2007). The protein moiety is coupled *via* its carboxy-terminus and an amide bond to this ethanolamine residue. In consequence, the protein moieties of GPI-anchored proteins are typically located at the surface of eucaryotic cells from yeast to man and can be released as soluble versions by cleavage with certain hydrolases, such as (G)PI-specific phospholipases C and D (Küng et al., 1997; Hoener et al., 1990; Müller et al., 1994a). Although the overall structure of GPI-anchors is highly conserved from yeast to man, they differ considerably with regard to their fatty acid and glycan compositions. The glycan variants from humans, funghi and protozoa differ with regard to their sugar constituents, side chains and glycosidic linkages (Nosjean et al., 1997; Ikezawa, 2002).

Transcellular transport of GPI-anchored proteins across adipocytes

During the past three years it became apparent that the GPI modification can operate as a signal for transcellular transport (Figure 3). These findings were initially obtained with adipocytes from young and old mice or rats. As is typical for all eukaryotic cells, also adipocytes express GPI-anchored proteins at their cell surface inserted into plasma membrane lipid rafts (Müller et al., 1994b; Varma and Major, 1998), i.e. detergent-insoluble glycolipid-enriched membrane microdomains (Brown and London, 1998). However unexpectedly, it was found that in primary and cultured rat and mouse adipocytes certain GPI-anchored proteins, among them the (c)AMP-binding and degrading proteins, Gce1 and CD73, are associated with both plasma membrane lipid rafts (major portion) and lipid droplets (minor

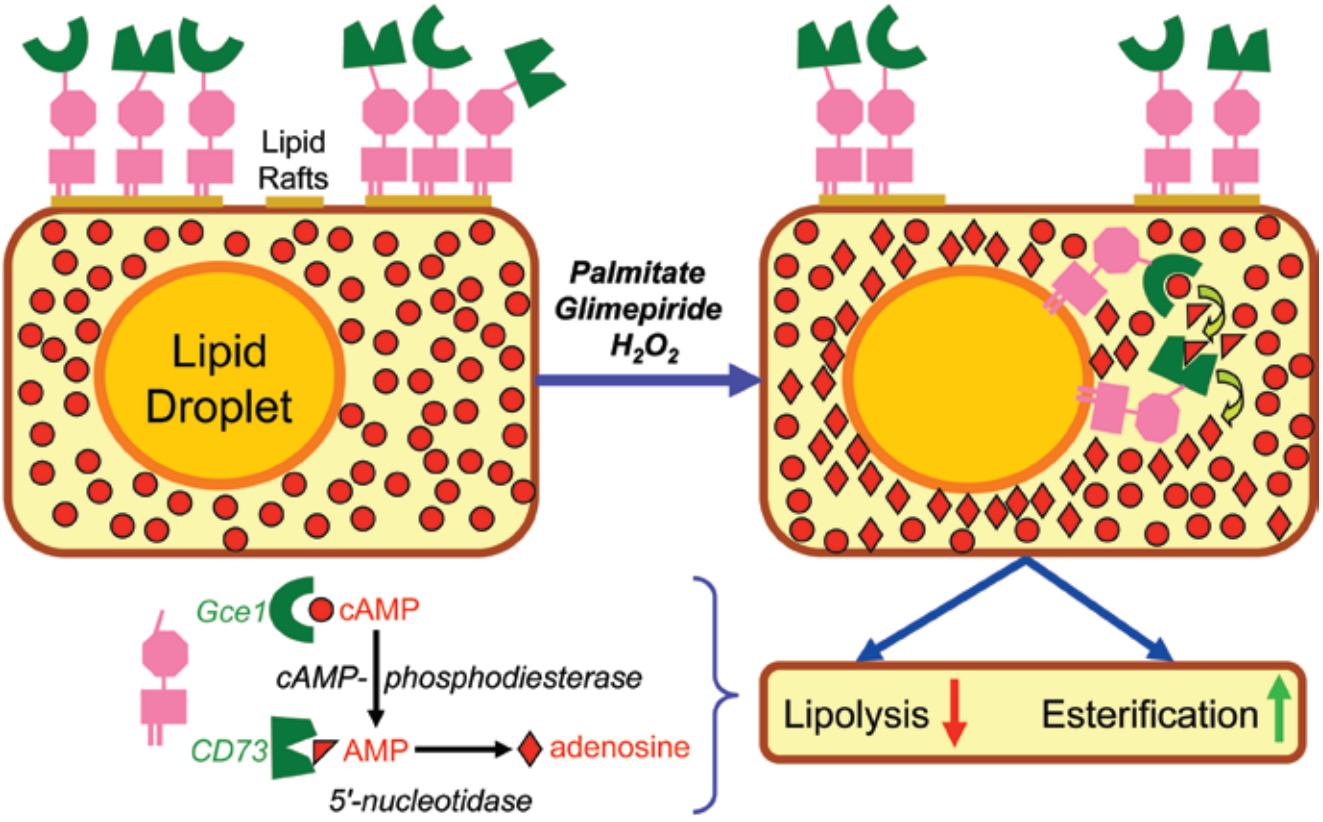

Figure 3. Model for the transport of GPI-anchored proteins from plasma membrane lipid rafts to intracellular lipid droplets in adipocytes. The GPI-anchored proteins, Gce1 and CD73, are transported from plasma membrane lipid rafts at the cell surface in the basal state to the surface of cytoplasmic lipid droplets upon challenge with palmitate, the anti-diabetic sulfonylurea drug glimepiride and hydrogen peroxide. Lowering of the cAMP concentration at the lipid droplet surface through phosphodiesteratic and nucleolytic cleavages by Gce1 and CD73, respectively, results in downregulation of lipolysis and upregulation of esterification in rodent adipocytes.

portion) (Müller et al., 2008a and b). These observations represented the first examples for intracellular residence and function of GPI-anchored proteins. Importantly this subcellular distribution of Gce1 and CD73 was detected in basal, i.e. unstimulated adipocytes. However, upon their challenge with certain seemingly unrelated stimuli, such as the anti-diabetic sulfonylurea drug glimepiride (Müller, 2005), hydrogen peroxide or palmitic acid, which all regulate glucose and lipid metabolism, the two GPI-anchored proteins completely disappeared from the plasma membranes and simultaneously appeared at the lipid droplets in time-dependent fashion (Müller et al., 2008c and d). This unequivocally demonstrated the stimulus-induced transport of Gce1 and CD73 from the cell surface onto the surface of cytoplasmic lipid droplets (Figure 3). As a prerequisite, the GPI-proteins have to undergo redistribution between distinct subspecies of the heterogenous plasma membrane lipid rafts, which are characterized by different flotation and solubilisation behaviour (Müller and Frick, 1999). Earlier observations with rat adipocytes have revealed the translocation of Gce1 from “typical” lipid rafts of high cholesterol content and low buoyant density to “atypical” ones of lower cholesterol content and higher buoyant density in response to glimepiride (Müller et al., 2001a and b), which was found to be required for its insulin-mimetic activities (Frick et al., 1998; Müller, 2005). Gce1 and CD73 cooperate in the degradation of cAMP through phosphodiesteratic cleavage by Gce1 to AMP and further to adenosine through nucleolytic cleavage by CD73. Thus the coordinated transport of Gce1 and CD73 from plasma membrane lipid rafts to cytoplasmic lipid droplets in response to palmitate, glimepiride and hydrogen peroxide results in lowering of the cAMP levels at the lipid droplet surface zone and consequently in the coordinated upregulation of esterification and downregulation of lipolysis in adipocytes (Müller et al., 2008e). This apparently represents (one of) the physiological role(s) of this unique transport pathway of Gce1 and CD73 in adipocytes (Figure 3).

Subsequently and unexpectedly, the transport of these GPI-proteins with major but not exclusive localization at the adipocyte cell surface was recognized not to stop at the surface of cytoplasmic lipid droplets. It is known since decades that almost each mammalian cell type is capable of releasing small membrane vesicles either by exocytosis, then they are called exosomes (Stoorvogel et al., 2002; Thery et al., 2002; Fevrier and Raposo, 2004; Keller et al., 2006), or by plasma membrane shedding or blebbing, then they are called microvesicles (Black, 1980; Poste and Nicolson, 1980; Piccin et al., 2007; Cocucci et al., 2009). Both exosomes and microvesicles are thought to reflect the

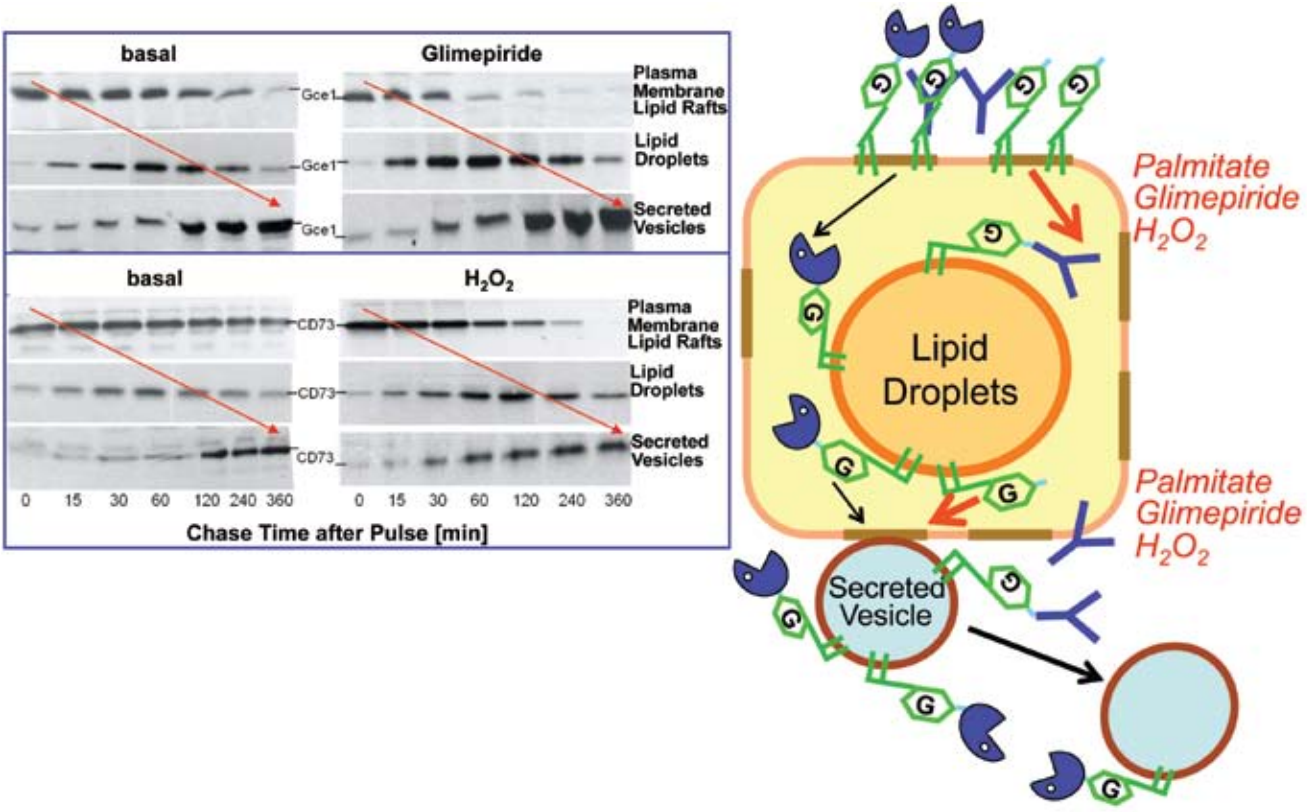

Figure 4. Model for the transcellular transport of GPI-anchored proteins across rat adipocytes as revealed by pulse-chase experiments. Palmitate, glimepiride and hydrogen peroxide trigger both the translocation of GPI-anchored proteins from the cell surface to the surface of cytoplasmic lipid droplets and subsequently from the lipid droplets into small secreted vesicles. This was delineated from pulse-labelling of the GPI anchors of Gce1 and CD73 with *myo*-[^{14}C]inositol and subsequent chase with unlabelled inositol in the presence of glimepiride or hydrogen peroxide. After initial localization at plasma membrane lipid rafts and subsequent transient appearance at lipid droplets, both Gce1 and CD73 finally accumulate in small secreted vesicles.

physiological or pathological state of the releasing cell and may be used as novel and putatively valuable biomarkers for the personalized diagnosis of common complex diseases. In addition, Aoki and coworkers (Aoki et al., 2007) and in the following our group too (Müller et al., 2009a and b) have recently found that also primary and cultured rat and mouse adipocytes release microvesicles and exosomes. Subsequently the GPI-anchored proteins, Gce1 and CD73, were identified as constituent components of those secreted vesicles, already in the basal state (Müller et al., 2009a and b). However, their incorporation into the secreted vesicles was strongly upregulated in response to glimepiride, hydrogen peroxide and palmitate (Figure 4). Thus the same stimuli that induce the translocation of these GPI-anchored proteins from plasma membrane lipid rafts to cytoplasmic lipid droplets trigger their subsequent translocation from the lipid droplets into membranes of small vesicles, which then leave the adipocytes. In fact, these two translocation steps in concert appear to mediate the transcellular transport of Gce1 and CD73 from the cell surface *via* cytoplasmic lipid droplets into the secreted vesicles. (Müller et al., 2010c and d).

Transcellular transport of GPI-anchored proteins across enterocytes

Is this transcellular transport of GPI-anchored proteins specific for adipocytes, only, or also operative in other mammalian cells, such as enterocytes? Preliminary data based on animal experiments argue for the last possibility (Müller et al., unpublished data). For this, human insulin covalently modified at its carboxy-terminus with the GPI anchor and produced by recombinant technologies was used as model protein. This GPI-anchored insulin was labelled with immunogold to enable visualization by electron microscopy. Following oral administration of the GPI-anchored insulin embedded in either micelles or liposomes to normal rats by gavage, the intestine was removed and sectioned. Subsequent analysis of the sections for immunogold-labelled GPI-anchored insulin by electron microscopy revealed rapid association of the GPI-anchored insulin with microvilli of the apical plasma membrane immediately upon its oral administration (Figure 5). The apparently spontaneous insertion of the GPI-anchored insulin into plasma membrane lipid rafts was further demonstrated by immunoblotting of intestinal subfractions of the plasma membrane with antibodies against human insulin and raft and non-raft proteins. Both GPI-anchored insulin and Gce1 was found to be highly enriched with lipid rafts compared to "non-lipid rafts" of the fractionated intestinal apical plasma membrane along with the corresponding marker proteins, the glucose transporter Glut2 and the fatty acid transporter CD36. Presumably, the GPI-anchored insulin became first inserted into lipid rafts of high cholesterol content and low

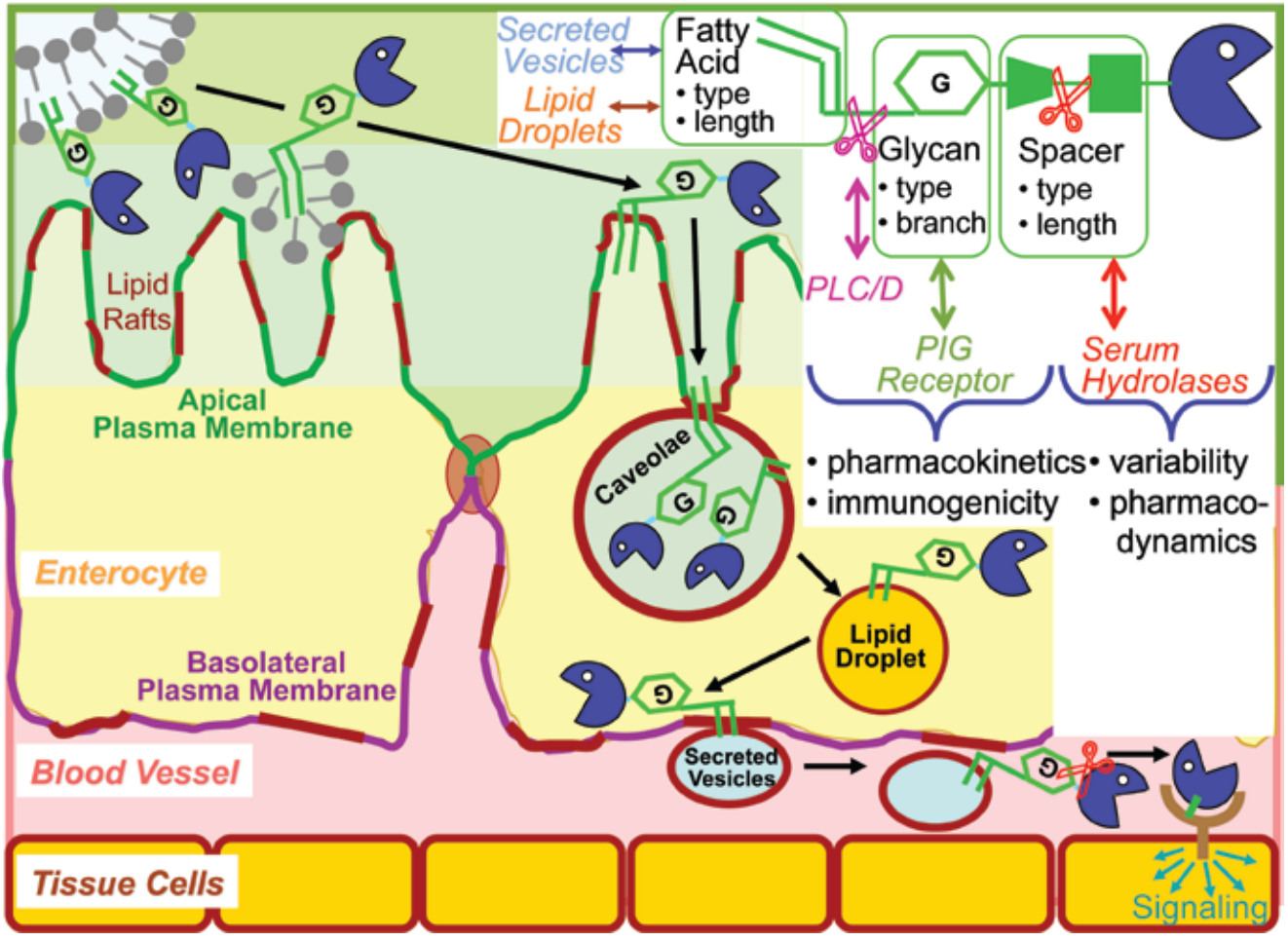

Figure 5. Model for the transcellular transport of GPI-anchored insulin across rat enterocytes as revealed by in vivo experiments and the structural determinants within the GPI anchor critical for the pharmacological profiles of GPI-anchored proteins. According to electron microscopic analyses of intestinal sections, following oral administration immunogold-labelled GPI-anchored insulin embedded in liposomes or micelles associates with lipid rafts of the apical plasma membrane, then becomes endocytosed *via* caveolae and later is inserted into the lipid droplet surface. Finally GPI-anchored insulin is transported across the basolateral plasma membrane and distributed *via* the circulation while bound to small secreted vesicles. Upon release from the GPI anchor by serum hydrolases, the free insulin binds to the insulin receptor of relevant tissue target cells which take up glucose from the blood. The inset indicates the multiple “pharmacological” roles of critical structural determinants of the GPI anchor and the underlying molecular mechanisms.

buoyant density, which are expressed in large amounts in enterocytes (Danielsen and Hansen, 2006 and 2008). Thereafter, it was redistributed within the outer plasma membrane leaflet to lipid rafts of lower cholesterol content and higher buoyant density prior to transport from these “atypical” lipid rafts to cytoplasmic lipid droplets (Müller et al., 2001a and b). Both spontaneous and protein-mediated incorporation of GPI-anchored proteins presented in micelles or liposomes or at the surface of donor cells into the surface of acceptor cells has been amply documented *in vitro* and *in vivo* (Zhang et al., 1992; McHugh et al., 1995; Ilangumara et al., 1996; Medof et al., 1996; Civenni et al., 1998; Kooyman et al., 1998; Nosjean et al., 1999; Suzuki and Okumura, 2000; Premkumar et al., 2001; Milhiet et al., 2002; Morandat et al., 2002; Ronzon et al., 2004). However, so far no therapeutic applications have been derived from this specific characteristic of GPI-anchored proteins.

Following the association with lipid rafts the immunogold-labelled GPI-anchored insulin was observed inside the enterocytes in the lumen of small closed membrane vesicles, presumably so-called caveolae (Lisanti et al., 1994; Mayor et al., 1994; Parton et al., 1994; Fivaz et al., 2002), in the immediate neighbourhood to the inner leaflet of the apical plasma membrane (Figure 5). Shortly thereafter the immunogold-labelled GPI-anchored insulin appeared at the surface of cytoplasmic lipid droplets, which are known to be expressed in enterocytes in large amounts (Zhu et al., 2009). Finally almost no immunogold-labelled GPI-anchored insulin was found left inside the enterocytes, which was taken as indication for its subsequent transport across the basolateral plasma membrane into the blood stream, possibly inserted into the membrane of secreted small vesicles. Eventually after being released from the vesicle surface through cleavage of the GPI anchor by

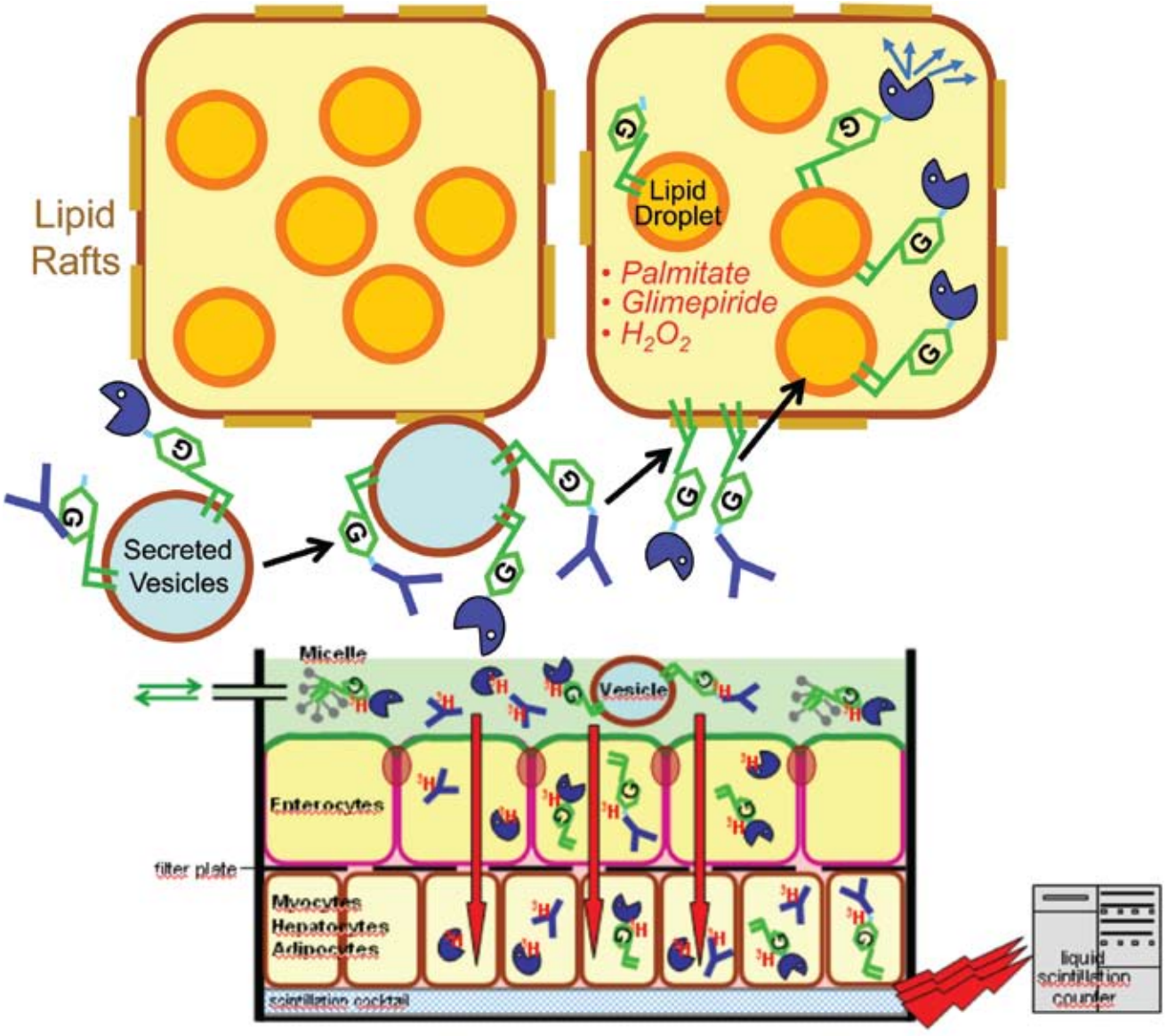

Figure 6. Model for the transport of GPI-anchored proteins from small secreted vesicles to lipid droplets in acceptor cells and its measurement by the modified multiplex assay system. GPI-anchored proteins are transferred from secreted vesicles to lipid rafts of acceptor adipocytes in constitutive fashion and thereafter are transported to the surface of lipid droplets in response to palmitate, glimepiride and hydrogen peroxide. The complete path of vesicle-associated, micelle-embedded or free radiolabelled GPI-anchored proteins across the intestinal and cellular barriers can be studied with a modified version of the multiplex assay system. It consists of two monolayers of adherent enterocyte and tissue target cells separated by a filter plate. Accumulation of radiolabelled GPI-anchored proteins in the tissue cell monolayer is monitored by liquid scintillation counting of the culture dish bottom.

serum phospholipases, free insulin could be distributed *via* the circulation to relevant target tissues, such as liver, muscle and fat, and exert its physiological action in course of binding to the insulin receptor (Figure 5).

Since the physiological role of insulin is lowering of blood glucose, this putative effect of orally administered GPI-anchored insulin was tested *in vivo* with insulin-free diabetic rats (Müller et al., unpublished results). As expected, oral administration of normal, i.e. unmodified human insulin by gavage did not result in detectable plasma insulin levels as well as lowering of the high blood glucose levels characteristic for these diabetic animals. In contrast, oral administration of GPI-anchored insulin produced in mammalian cell culture provoked dramatic increases in plasma insulin and in parallel pronounced decreases in blood glucose. As a control for the role of the GPI modification, the oral administration of GPI-anchored insulin from which the GPI anchor had been removed by bacterial phospholipase C cleavage was completely ineffective. In conclusion, GPI-anchored insulin is apparently transported from the intestinal lumen across the enterocytes into the circulation and upon distribution to liver, muscle and adipose tissues and subsequent release from the GPI anchor or vice versa triggers insulin signalling which results in blood glucose decrease.

These proof-of-concept experiments were then repeated with GPI-anchored insulin produced by two different yeast strains rather than in mammalian cell culture (Müller et al., unpublished data). Unexpectedly, the time-action profiles of the three GPI-anchored insulins, which differed only with regard to the structure of the glycan cores of their GPI anchors exhibited significant differences. The "fungal" GPI-anchored insulins produced by *Pichia pastoris* and *Saccharomyces cerevisiae* induced very rapid and delayed onsets, respectively, of the plasma insulin increases compared to that elicited by the "mammalian" GPI-anchored insulin. The time courses of the plasma insulin increases were correlated well to the corresponded blood glucose decreases. Thus apparently the composition of the glycan core determines the pharmacokinetic profile of the GPI-anchored insulin, at least in part, which presumably relies on its interaction with the recently identified phosphoinositolglycan (PIG) receptor (Müller et al., 2002a and b) and on its accessibility to cleavage by the serum phospholipases C/D (Bütikofer and Brodbeck, 1993; Küng et al., 1997; Müller et al., 2005). Moreover, the glycan core harbours intrinsic immunogenic potential (Figure 5). Unfortunately, the complexity increases further since considerable variation in the time-action profiles for a given GPI-anchored insulin was observed between different animals in the same experiment as well as between different experiments with the same animal (Müller et al., unpublished data). These deviations were, at least in part, caused by both intra-individual and inter-individual variability in the concentrations of the serum hydrolases cleaving off the passenger protein from the GPI anchor within the spacer element. This spacer which separates the passenger, here insulin, from the anchor is thus critical for the pharmacodynamic profile and its variability elicited by a therapeutic GPI-anchored protein (Figure 5). Systematic variation of all structural elements of the GPI anchor by genetic manipulation and expression in different microorganisms, as already shown for *S. cerevisiae* and *P. pastoris*, will be crucial for their fine mapping. No doubt, these data will support the engineering of novel therapeutic proteins and antibodies with the crucial advantages of oral administration, selective targeting to and into the relevant diseased target cells and curing their defects in the underlying susceptibility gene products with the desired pharmacokinetic and pharmacodynamic profiles.

Intercellular transport of GPI-anchored proteins by small secreted vesicles

The journey of GPI-anchored proteins having successfully crossed the intestinal barrier and remaining associated with the small secreted vesicles *via* their intact GPI anchor eventually does not terminate in the circulation. It has recently been reported (Müller et al., 2010c and d) that upon incubation of small vesicles secreted from "donor" adipocytes with intact "acceptor" adipocytes, the constituent GPI-anchored proteins, such as Gce1 and CD73, are being transferred from the small secreted vesicles to plasma membrane lipid rafts of the "acceptor" adipocytes, presumably in course of their direct interaction (Figure 6) Müller et al., 2010c and d). Subsequently, the journey of the transferred GPI-anchored proteins is continued within the "acceptor" adipocytes by their transport from the outer leaflet of the plasma membrane lipid rafts to cytoplasmic lipid droplets. This again was found to be stimulated by palmitate, glimepiride and hydrogen peroxide (Müller et al., 2010a and b).

Thus, the small secreted vesicles with the inserted GPI-anchored proteins do not only operate as "passive" vehicles for their distribution to target tissues *via* the circulation. They also play a more "active" role in tissue distribution of GPI-anchored proteins by mediating their passage across the plasma membrane into the cytoplasm of the "acceptor", i.e. target cell. This is compatible with recent findings that the type of the fatty moieties of the GPI anchor (i.e. length, saturation) critically contributes to the distribution of the GPI-anchored protein between plasma and tissues, between distinct tissues and between subcellular compartments (Müller et al., unpublished data). This is due to, at least in part, mediation of the interaction of the GPI-anchored protein with lipid raft-like membrane microdomains (Brown, 1992) of small secreted vesicles as well as of cytoplasmic lipid droplets by the GPI anchor fatty acyl chains (Figure 6).

It will be an important and challenging task for the future to elucidate in detail all the structural features of the GPI anchor which determine the pharmacokinetic and pharmacodynamic profiles as well as putative immunological and toxic properties of GPI-anchored proteins for the realization and optimization of their oral application in humans. Part of the desired information may be derived from *in vitro* investigations using a modified version (Figure 5) of the multiplex assay system described above (see Figure 2). For this, the filter plate with the adherent sealed enterocyte monolayer at the top is placed above an additional sealed monolayer of putative target tissue cells, such as hepatocytes, myocytes and adipocytes. The filter plate prevents direct cell-to-cell contacts between the two monolayers, but allows the exchange of small molecules, such as nutrients and ions. In the bottom of the culture dish to which the target tissue cells adhere liquid scintillation cocktail is incorporated (Figure 5). The transport of vesicle-, liposome- or micelle-associated radiolabelled GPI-anchored proteins applied to the upper apical area across the enterocytes *via* the fluid space formed by the pores of the filter plate and representing the basolateral area into the underlying target tissue cells is monitored as accumulation of radioactivity in the monolayer cells adherent to the bottom of the culture dishes by liquid scintillation counting.

Conclusions

The intention of this review was to introduce two novel strategies for oral delivery and tissue and intracellular targeting of therapeutic proteins and antibodies. They should facilitate the future development of personalized polypharmacy based on multiple and individual combinations of orally available protein drugs for correction of the multiple susceptibility genes defective in common complex diseases. Meanwhile the successful sequencing of the collective genome of all our resident microorganisms, the human microbiome (Nelson et al., 2010; Zhao, 2010), may support the identification of novel microbial biomaterials appropriate for nanoparticle formation and protein modifications enabling transcellular transport with potential for therapeutic application in humans. Ultimately, all these strategies should overcome the limitations inherent of injection of a single and the same drug, only, to all patients affected by the same common complex disease. This is best exemplified by the current non-oral monopharmacy of type II diabetes with long-lasting insulin derivatives.

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References

Aoki,N., Jin-no,S., Nakagawa,Y., Asai,N., Arakawa,E., and Tamura,N. (2007). Identification and characterization of microvesicles secreted by 3T3-L1 adipocytes: redox- and hormone-dependent induction of milk fat globule-epidermal growth factor 8-associated microvesicles. Endocrinology *148*, 3850-3862.

Billingsley,M.L. (2008). Druggable targets and targeted drugs: enhancing the development of new therapeutics. Pharmacology *82*, 239-244.

Black,P.N. (1980). Shedding from normal and cancer-cell surfaces. N. Engl. J. Med. *303*, 1415-1420.

Brewis,I.A., Ferguson,M.A.J., Mehlert,A., Turner,A.J., and Hooper,N.M. (1995). Structures of the glycosyl-phosphatidylinositol anchors of porcine and human eythrocyte renal membrane dipeptidase. Comprehensive structural studies on the porcine anchor and interspecies comparison of the glycan core structures. J. Biol. Chem. *270*, 22946-22956.

Brown,D.A. (1992). Interactions between GPI-anchored proteins and membrane lipids. Trends Cell Biol. *2*, 338-343.

Brown,D.A., and London,L. (1998). Functions of lipid rafts in biological membranes. Annu. Rev. Cell. Dev. Biol. *14*, 111-136.

Bütikofer,P., and Brodbeck,U. (1993). Partial purification and characterization of a (glycosyl) inositol phospholipid-specific phospholipase C from peanut. J. Biol. Chem. *268*, 17794-17799.

Civenni,G., Test,S.T., Brodbeck,U., and Bütikofer,P. (1998). In vitro incorporation of GPI-anchored proteins into human erythrocytes and their fate in the membrane. Blood *9*, 11784-11792.

Cocucci,E., Racchetti,G., and Meldolesi,J. (2009). Shedding microvesicles: artefacts no more. Trends Cell Biol. *19*, 43-51.

Danielsen,E.M., and Hansen,G.H. (2008). Lipid raft organization and function in the small intestinal brush border. J. Physiol. Biochem. *64*, 377-382.

Danielsen,E.M., and Hansen,G.H. (2006). Lipid raft organization and function in brush borders of epithelial cells. Mol. Membr. Biol. *23*, 71-79.

Ebbesen,M., and Jensen,T.G. (2006). Nanomedicine: techniques, potentials, and ethical implications. J. Biomed. Biotechnol. DOI10.1155/JBB/2006/51516.

Fevrier,B., and Raposo,G. (2004). Exosomes: endosomal-derived vesicles shipping extracellular messages. Curr. Opin. Cell Biol. *16*, 415-421.

Fivaz,M., Vilbois,F., Thurnheer,S., Pasquali,C., Abrami,L., Bickel,P.E., Parton,R.G., van der Goot,F.G. (2002). Differential sorting and fate of endocytosed GPI-anchored proteins. EMBO J. *21*, 3989-4000.

Florez,J.C. (2008). The genetics of type 2 diabetes: a realistic appraisal in 2008. J. Clin. Endocrinol. Metab. *93*, 4633-4642.

Gibson,G. (2009). Decanalization and the origin of complex disease. Nature *10*, 134-140.

Gullotti,E., and Yeo,Y. (2009). Extracellularly activated nanocarriers: A new paradigm of tumor targeted drug delivery. Mol. Pharmaceut. *6*, 1041-1051.

Hoener,M.C., Stieger,S., and Brodbeck,U. (1990). Isolation and characterization of a phosphatidylinositol-glycan-anchor-specific phospholipase D from bovine brain. Eur. J. Biochem. *190*, 593-600.

Ikezawa,H. (2002). Glyosylphosphatidylinositol (GPI)-anchored proteins. Biol. Pharm. Bull. *25*, 409-417.

Ilangumaran,S., Robinson,P.J., and Hoessli,D.C. (1996). Transfer of exogenous glycosylinositol (GPI)-linked molecules to plasma membranes. Trends Cell Biol. *6*, 163-169.

Janssens,A.C.J.W., Pardo,M.C., Steyerberg,E.W., and van Dujin,C.M. (2004). Revisiting the clinical validity of multiplex genetic testing in complex diseases. Am. J. Hum. Genet. *74*, 585-588.

Keller,S., Sanderson,M.P., Stoeck,A., and Altevogt,P. (2006). Exosomes: from biogenesis and secretion to biological function. Immunol. Lett. *107*, 102-108.

Kirpotin,D.B., Drummond,D.C., Shao,Y., Shalaby,M.R., Hong,K.L., Nielsen,U.B., Marks,J.D., Benz,C.C., and Park,J.W. (2006). Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models. Cancer Res. *66*, 6732-6740.

Kooyman,D.L., Byrne,G.W., and Logan,J.S. (1998). Glycosyl phosphatidylinositol anchor. Exp. Nephrol. *6*, 148-151.

Küng,M., Bütikofer,P., Brodbeck,U., and Stadelmann,B. (1997). Expression of intracellular and GPI-anchored forms of GPI-specific phospholipase D in COS-1 cells. Biochem. Biophys. Acta *1357*, 329-338.

Li,S.D., and Huang,L. (2007). Non-viral is superior to viral gene delivery. J. Controlled Release *123*, 181-183.

Lin,Y.H., Mi,F.L., Chen,C.T., Chang,W.C., Peng,S.F., Liang,H.F., and Sung,H.W. (2007). Preparation and characterization of nanoparticles shelled with chitosan for oral insulin delivery. Biomacromolecules *8*, 146-152.

Lindgren,C.M. (2007). Mechanisms of disease: genetic insights into the etiology of type 2 diabetes and obesity. Nature Clin. Pract. *4*, 156-163.

Lisanti,M.P., Scherer,P.E., Tang,Z.L., and Sargiacomo,M. (1994). Caveolae, caveolin and caveolin-rich membrane domains: A signalling hypothesis. Trends Cell Biol. *4*, 231-238.

Lusis,A.J., Attie,A.D., and Reue,K. (2008). Metabolic syndrome: from epidemiology to systems biology. Nature Genet. *9*, 819-830.

Mathiowitz,E. (2008). Drug delivery system. Toxicol. Pathol. *36*, 16-22.

Mathiowitz,E., Jacob,J.S., Jong,Y.S., Carino,G.P., Chickering,D.E., Chaturvedi,P., Santos,C.A., Vijayaraghavan,K., Montgomery,S., Bassett,M., and Morrell,C. (1997). Biologically erodable microspheres as potential oral drug delivery systems. Nature *386*, 410-414.

Matsumara,Y. (2008). Polymeric micellar delivery systems in oncology. Jpn. J. Clin. Oncology *38*, 793-802.

Mayor,S., Rothberg,K.G., and Maxfield,F.R. (1994). Sequestration of GPI-anchor proteins in caveolae triggered by cross-linking. Science *264*, 1948-1951.

McHugh,R.S., Ahmed,S.N., Wang,Y.C., Sell,K.W., and Selvavaj, P. (1995). Construction, purification, and functional incorporation on tumor cells of glycolipid-anchored human B7-1 (CD80). Proc. Natl. Acad. Sci. USA *92*, 8059-8063.

Medof,M.E., Nagarajan,S., and Tykocinski,M.L. (1996). Cell-surface engineering with GPI-anchored proteins. FASEB J. *10*, 574-586.

Milhiet,P.-E., Giocondi,M.-C., Baghdadi,O., Ronzon,F., Roux,B., and Le Grimellec,C. (2002). Spontaneous insertion and partitioning of alkaline phosphatase into model lipid rafts. EMBO reports *3*, 485-490.

Mok,H., Bae,K.H., Ahn,C.-H., and Park,T.G. (2009). PEGylated and MMP-2 specifically dePEGylated quantum dots: Comparative evaluation of cellular uptake. Langmuir *25*, 1645-1650.

Morandat,S., Bortolato,M., and Roux,B. (2002). Cholesterol-dependent insertion of glycosylphosphatidylinositol-anchored enzyme. Biochim. Biophys. Acta *1564*, 473-478.

Müller,G. (2005). The mode of action of glimepiride – beyond insulin secretion. Curr. Med. Chem. *5*, 499-518.

Müller,G. (2010). Personalized prognosis and diagnosis of type II diabetes - vision or fiction? Pharmacology *85*, 168-187.

Müller,G., and Frick,W. (1999). Signalling via caveolin: Involvement in the cross-talk between phosphoinositolglycans and insulin. Cell. Mol. Life Sci. *56*, 945-970.

Müller,G., Dearey,E.-A., Korndörfer,A., and Bandlow,W. (1994a). Stimulation of a glycosyl-phosphatidylinositol-specific phospholipase by insulin and the sulfonylurea, glimepiride, in rat adipocytes depends on increased glucose transport. J. Cell Biol. *126*, 1267-1276.

Müller,G., Wetekam,E.-M., Jung,C., and Bandlow,W. (1994b). Membrane association of lipoprotein lipase and a cAMP-binding ectoprotein in rat adipocytes. Biochemistry *33*, 12149-12159.

Müller,G., Jung,C., Wied,S., Welte,S., and Frick,W. (2001a). Insulin-mimetic signaling by the sulfonylurea glimepiride and phosphoinositolglycans involves distinct mechanisms for redistribution of lipid raft components. Biochemistry *40*, 14603-14620.

Müller,G., Jung,C., Wied,S., Welte,S., Jordan,H., and Frick,W. (2001b). Redistribution of glycolipid raft domain components induces insulin-mimetic signaling in rat adipocytes. Mol. Cell. Biol. *21*, 4553-4567.

Müller,G., Hanekop,N., Kramer,W., Bandlow,W., and Frick,W. (2002a). Interaction of phosphoinositolglycan(-peptides) with plasma membrane lipid rafts of rat adipocytes. Arch. Biochem. Biophys. *408*, 17-32.

Müller,G., Jung,C., Frick,W., Bandlow,W., and Kramer,W. (2002b). Interaction of phosphoinositolglycan(-peptides) with plasma membrane lipid rafts triggers insulin-mimetic signaling in rat adipocytes. Arch. Biochem. Biophys. *408*, 7-16.

Müller,G., Schulz,A., Wied,S., and Frick,W. (2005). Regulation of lipid raft proteins by glimepiride- and insulin-induced glycosylphosphatidylinositol-specific phospholipase C in rat adipocytes. Biochem. Pharmacol. *69*, 761-780.

Müller,G., Jung,C., and Wied,S. (2008a). Translocation of glycosylphosphatidylinositol-anchored proteins from plasma membrane microdomains to lipid droplets in rat adipocytes is induced by palmitate, H_2O_2 and the sulfonylurea drug, glimepiride. Mol. Pharmacol. *73*, 1513-1529.

Müller,G., Over,S., Wied,S., and Frick,W. (2008b). Association of (c)AMP-degrading glycosylphosphatidylinositol-anchored proteins with lipid droplets is induced by palmitate, H_2O_2 and the sulfonylurea drug, glimepiride, in rat adipocytes. Biochemistry *47*, 1274-1287.

Müller,G., Wied,S., Jung,C., and Over,S. (2008c). Translocation of glycosylphosphatidylinositol-anchored proteins to lipid droplets and inhibition of lipolysis in rat adipocytes is mediated by reactive oxygen species. Br. J. Pharmacol. *154*, 901-913.

Müller,G., Wied,S., Jung,C., and Straub,J. (2008d). Coordinated regulation of esterification and lipolysis by palmitate, H_2O_2 and the anti-diabetic sulfonylurea drug, glimepiride, in rat adipocytes. Eur. J. Pharmacol. *597*, 6-18.

Müller,G., Wied,S., Over,S., and Frick,W. (2008e). Inhibition of lipolysis by palmitate, H_2O_2 and the sulfonylurea drug, glimepiride, in rat adipocytes depends on cAMP degradation by lipid droplets. Biochemistry *47*, 1259-1273.

Müller,G., Jung,C., Straub,J., Wied,S., and Kramer,W. (2009a). Induced release of membrane vesicles from rat adipocytes containing glycosylphosphatidylinositol-anchored microdomain and lipid droplet signalling proteins. Cell. Signal. *21*, 324-338.

Müller,G., Jung,C., Wied,S., and Biemer-Daub,G. (2009b). Induced translocation of glycosylphosphatidylinositol-anchored proteins from lipid droplets to adiposomes in rat adipocytes. Br. J. Pharmacol. *158*, 749-770.

Müller,G., Schulz,A., Dearey,E.-A., Wetekam,E.-M., Wied,S., and Frick,W. (2010a). Synthetic phosphoinositolglycans regulate lipid metabolism between rat adipocytes *via* release of GPI-protein-harbouring adiposomes. Arch. Physiol. Biochem., *116*, 97-115.

Müller,G., Schulz,A., Hartz,D., Dearey,E.-A., Wetekam,E.-M., Ökonomopulos,R., Crecelius,A., Wied,S., and Frick,W. (2010b). Novel glimepiride derivatives with potential as double-edged swords against type II diabetes? Arch. Physiol. Biochem. *116*, 3-20.

Müller,G., Wied,S., Jung,C., Biemer-Daub,G., and Frick,W. (2010c). Transfer of glycosylphosphatidylinositol-anchored 5'-nucleotidase CD73 from adiposomes into rat adipocytes stimulates lipid synthesis. Br. J. Pharmacol. *160*, 878-891.

Müller,G., Wied,S., Jung,C., Frick,W., and Biemer-Daub,G. (2010d). Inhibition of lipolysis by adiposomes containing glycosylphosphatidylinositol-anchored Gce1 protein in rat adipocytes. Arch. Physiol. Biochem. *116*, 28-41.

Nelson,K.E., and The Human Microbiome Jumpstart Reference Strains Consortium. (2010). A Catalog of Reference Genomes from the Human Microbiome. Science *328*, 994-999.

Nosjean,O., Briolay,A., and Roux,B. (1997). Mammalian GPI proteins: sorting, membrane residence and functions. Biochim. Biophys. Acta *1331*, 153-186.

Nosjean,O., and Roux, B. (1999). Ectoplasmic insertion of a glycosylphosphatidylinositol-anchored protein in glycosphingolipid- and cholesterol-containing phosphatidylcholine vesicles. Eur. J. Biochem. *263*, 865-870.

Orlean,P., and Menon,A.K. (2007). Thematic review series: lipid posttranslational modifications. GPI anchoring of protein in yeast and mammalian cells, or: how we learned to stop worrying and love glycophospholipids. J. Lipid Res. *48*, 993-1011.

Parton,R.G., Joggerst,B., and Simons,K. (1994). Regulated internalization of caveolae. J. Cell Biol. *127*, 1199-1215.

Piccin,A., Murphy,W.G., and Smith,O.P. (2007). Circulating microparticles: pathophysiology and clinical implications. Blood Rev. *21*, 157-171.

Poste,G., and Nicolson,G.L. (1980). Arrest and metastasis of blood-born tumor cells are modified by fusion of plasma membrane vesicles from highly metastatic cells. Proc. Natl. Acad. Sci. USA *77*, 399-403.

Premkumar,D.R., Fukuoka,Y., Sevlever,D., Brunschwig,E., Rosenberry,T.L., and Tykocinski,M.L. (2001). Properties of exogenously added GPI-anchored proteins following their incorporation into cells. J. Cell. Biochem. *82*, 234-245.

Prokopenko,I., McCarthy,M.J., and Lindgren,C.M. (2008). Type 2 diabetes: new genes, new understanding. Trends Genet. *24*, 613-621.

Rajendran,L., Knölker,H.-J., and Simons,K. (2010). Subcellular targeting strategies for drug design and delivery. Nature Rev. Drug Discovery *9*, 29-42.

Ridderstrale,M., and Groop,L. (2009). Genetic dissection of type 2 diabetes. Mol. Cell. Endocrinol. *297*, 10-17.

Ronzon,F., Morandat,S., Roux,B., and Bortolato,M. (2004). Insertion of a glycosylphosphatidylinositol-anchored enzyme into liposomes. J. Membrane Biol. *197*, 169-177.

Soussan,E., Cassel,S., Blanzat,M., and Rico-Lattes,I. (2009). Drug delivery by soft matter: Matrix and vesicular carriers. Angew. Chem. Int. Ed. *48*, 274-288.

Stoorvogel,W., Kleijmeer,M.J., Geuze,H., and Raposo,G. (2002). The biogenesis and functions of exosomes. Traffic *3*, 321-330.

Suzuki,K., and Okumura,Y. (2000). GPI-linked proteins do not transfer spontaneously from erythrocytes to liposomes. New aspects of reorganization of the cell membrane. Biochemistry *39*, 9477-9485.

Thery,C., Zitvogel,L., and Amigorena,S. (2002). Exosomes: composition, biogenesis and function. Nat. Rev. Immunol. *3*, 569-579.

Uhrich,K.E., Cannizzaro,S.M., Langer,R.S., and Shakesheff,K.M. (1999). Polymeric systems for controlled drug release. Chem. Rev. *99*, 3181-3198.

Varma,R., and Mayor,S. (1998). GPI-anchored proteins are organized in submicron domains at the cell surface. Nature *394*, 798-801.

Yang,T., Choi,M.K., Cui,F.D., Kim,J.S., Chung,S.J., Shim,C.K., and Kim,D.D. (2007). Preparation and evaluation of paclitaxel-loaded PEGylated immunoliposome. J. Controlled Release *120*, 169-177.

Yokoyama,M. (2005). Drug targeting with nano-sized carrier systems. J. Artif. Organs *8*, 77-84.

Zhang,F., Schmidt,W.G., Hou,Y., Williams,A.F., and Jacobson,K. (1992). Spontaneous incorporation of the glycosyl-phosphatidylinositol-linked protein Thy-1 into cell membranes. Proc. Natl. Acad. Sci. USA *89*, 5231-5235.

Zhao,L. (2010). The tale of our other genome. Science *465*, 879-880.

Zhu,J., Lee,B., Buhman,K.K., and Cheng,J.X. (2009). A dynamic, cytoplasmic triacylglycerol pool in enterocytes revealed by ex vivo and in vivo coherent anti-Stokes Raman scattering imaging. J. Lipid Res. *59*, 1080-1089.

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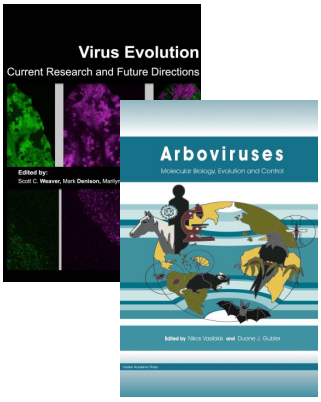

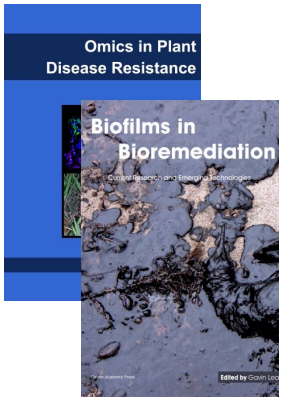

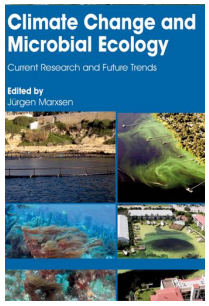

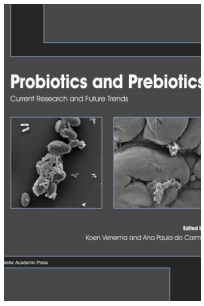

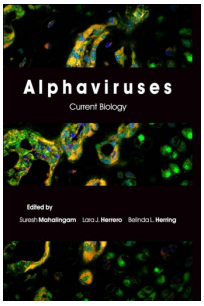

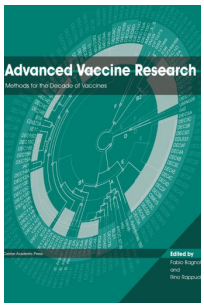

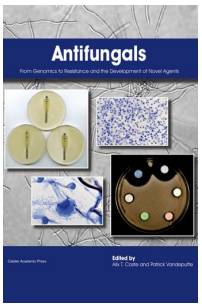

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