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Functional Study of the Vitamin K Cycle Enzymes in Live Cells

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Abstract

Vitamin K-dependent carboxylation, an essential posttranslational modification catalyzed by gamma-glutamyl carboxylase, is required for the biological functions of proteins that control blood coagulation, vascular calcification, bone metabolism, and other important physiological processes. Concomitant with carboxylation, reduced vitamin K (KH₂) is oxidized to vitamin K epoxide (KO). KO must be recycled back to KH₂ by the enzymes vitamin K epoxide reductase and vitamin K reductase in a pathway known as the vitamin K cycle. Our current knowledge about the enzymes of the vitamin K cycle is mainly based on *in vitro* studies of each individual enzymes under artificial conditions, which are of limited usefulness in understanding how the complex carboxylation process is carried out in the physiological environment. In this chapter, we review the current *in vitro* activity assays for vitamin K cycle enzymes. We describe the rationale, establishment, and application of cell-based assays for the functional study of these enzymes in the native cellular milieu. In these cell-based assays, different vitamin K-dependent proteins were designed and stably expressed in mammalian cells as reporter proteins to accommodate the readily used enzyme-linked immunosorbent assay for carboxylation efficiency evaluation. Additionally, recently emerged genome-editing techniques TALENs and CRISPR-Cas9 were used to knock out the endogenous enzymes in the reporter cell lines to eliminate the background. These cell-based assays are easy to scale up for high-throughput screening of inhibitors of vitamin K cycle enzymes and have been successfully used to clarify the genotypes and their clinical phenotypes of enzymes of the vitamin K cycle.

1. INTRODUCTION

Vitamin K-dependent (VKD) carboxylation is an essential posttranslational modification that converts certain glutamate (Glu) residues to gamma-carboxyglutamate (Gla) residues in VKD proteins. The modification involves the addition of a carboxyl group to the gamma carbon of Glu residues in the VKD protein. Carboxylation is catalyzed by the enzyme gamma-glutamyl carboxylase (GGCX), which utilizes a reduced form of vitamin K (vitamin K hydroquinone, or KH₂), carbon dioxide, and oxygen as cofactors. Concomitant with each Glu modification, KH₂ is oxidized to vitamin K 2,3-epoxide (KO). Since vitamin K is rapidly catabolized in the human body (Shearer & Newman, 2014), KO must be reused and is converted back to KH₂ through a two-step reduction by the enzymes vitamin K epoxide reductase (VKOR) and the as-yet-unknown vitamin K reductase (VKR) in a pathway known as the vitamin K cycle (Fig. 1). Because of the hydrophobic characteristics of vitamin K,

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enzymes in the vitamin K cycle are likely integral membrane proteins that reside in the endoplasmic reticulum (ER).

VKD carboxylation has been associated mostly with coagulation, because it was originally observed in clotting factors (Nelsestuen, Zytkevich, & Howard, 1974; Stenflo, Fernlund, Egan, & Roepstorff, 1974). All the VKD clotting factors require the 9–13 Glu residues at the N-terminus of the protein to be fully carboxylated in order for the protein to be functional. Defects of VKD carboxylation have long been known to cause bleeding disorders, known as combined vitamin K-dependent coagulation factors deficiency (VKCFD; Napolitano, Mariani, & Lapecorella, 2010; Prentice, 1985). With the discovery of new Gla proteins, the importance of VKD carboxylation has been extended beyond coagulation into a number of other physiological functions. For example, carboxylated matrix Gla protein (MGP) is a strong inhibitor of vascular calcification and of connective tissue mineralization, and uncarboxylated MGP has been implicated in cardiovascular diseases and other nonbleeding syndromes (Willems, Vermeer, Reutelingsperger, & Schurgers, 2014). Another VKD protein, osteocalcin (also called bone Gla protein, or BGP), is produced by osteoblasts and is important for bone formation (Ducy et al., 1996); recent studies suggest that osteocalcin also functions as a hormone affecting glucose metabolism (Mera et al., 2016). Therefore, functional study of the vitamin K cycle enzymes is not only essential for understanding blood coagulation, but it is also important for understanding many other physiological processes.

1.1 Functional Study of VKOR by *In Vitro* Activity Assay

The enzymatic activity of VKOR was discovered in 1970 (Bell & Matschiner, 1970). These authors showed that vitamin K-deficient rats can efficiently reduce KO to vitamin K and that this activity is sensitive to warfarin inhibition. Results from rat liver homogenates showed that the enzymatic activity of VKOR was enriched in purified microsomes (Zimmermann & Matschiner, 1974). However, fresh purified microsomes are inactive unless a thiol reagent, such as dithiothreitol (DTT), is included, suggesting that the thiol reagent is an artificial electron donor for VKOR activation. Based on a chemical model study, Silverman proposed that DTT reduces a critical disulfide bond within the active site of VKOR, and that the reduced cysteines are reoxidized to a disulfide during the reduction of KO to vitamin K (Silverman, 1981). Dithiol reagents were found to be more efficient than monothiol reagents as reductants for VKOR enzymatic activity (Lee & Fasco, 1984). The authors of this study suggested that monothiols are inefficient because a major proportion of the mixed disulfide, initially formed by reaction between the monothiol and the VKOR disulfide, undergoes reoxidation before a second molecule of monothiol can react. With the identification of the VKOR gene and functional studies of VKOR at the molecular level, we now know that Cys132 and Cys135 are the active site residues that form a disulfide after the reduction of KO (Li et al., 2004; Rost et al., 2004; Wajih, Hutson, Owen, & Wallin, 2005).

The need to reduce an active site disulfide of VKOR to initiate its enzymatic activity stimulated the search for the physiological reductant of VKOR (Silverman & Nandi, 1988). Thijssen et al. reported that rat liver microsomes with VKOR also contain NADH-dependent lipoamide reductase activity (Thijssen, Janssen, & Vervoort, 1994). The reduced lipoamide

stimulates microsomal VKOR activity with kinetics comparable to those of DTT. They proposed that microsomal lipoamide reductase is associated with VKOR in the membrane and is capable of transferring the reducing equivalents from NADH via reduced lipoamide to VKOR (Thijssen et al., 1994). However, a vast body of evidence suggests that thioredoxin is the physiological reductant of VKOR (Gardill & Suttie, 1990; Johan, van Haarlem, Soute, & Vermeer, 1987; Silverman & Nandi, 1988). Results from Soute et al. show that the protein disulfide isomerase (PDI) enhances the thioredoxin-driven VKOR activity ~ 10-fold (Soute, Groenen-van Dooren, Holmgren, Lundstrom, & Vermeer, 1992). However, this hypothesis has been questioned by Preusch (1992), because thioredoxin is predominantly a soluble cytoplasmic protein that would not have access to the luminal surface of the ER where VKOR's active site is located. Based on results showing that reduced RNAase can trigger PDI-dependent VKD carboxylation, and that this activity was inhibited by the PDI inhibitor bacitracin and by siRNA silencing of PDI, Wajih et al. proposed that PDI can directly provide electrons for the reduction of VKOR's active site (Wajih, Hutson, & Wallin, 2007). Recently, by scanning a large number of mammalian thioredoxin-like ER proteins, including PDI, Schulman et al. reported that VKOR prefers the membrane-bound thioredoxin-like redox partners TMX (Schulman, Wang, Li, & Rapoport, 2010). Despite decades of effort, the physiological reductant of VKOR is still unknown.

In the absence of a confirmed physiological reductant for VKOR, DTT is used as the reductant in the standard VKOR *in vitro* activity assay. This type of assay was first performed under a nitrogen atmosphere by blowing nitrogen over the incubation mixture (Whitlon, Sadowski, & Suttie, 1978; Zimmermann & Matschiner, 1974). The reaction was started by the addition of ³H-labeled KO and stopped by the addition of two volumes of an organic solvent, such as isopropanol:hexane (3:2). The radioactive K vitamins in the hexane extract were separated by reversed-phase silica-based thin-layer chromatography. KO and vitamin K spots were cut from the plate, placed in counting vials with scintillation fluid, and quantified by measuring the radioactivity with a liquid scintillation counter (Matschiner, Bell, Amelotti, & Knauer, 1970).

Because of its complexity and its use of radioactive vitamin K, this type of VKOR activity assay was soon replaced; newer assays were performed using reverse-phase HPLC to separate the reactant and product of K vitamins, which were measured using UV detection (Elliott, Townsend, & Odam, 1980; Fasco & Principe, 1980). Typically, the reaction was performed with nonlabeled KO; after stopping the reaction with organic solvent, the vitamin K-containing organic phase was evaporated, redissolved, and applied to a reverse-phase HPLC using isocratic elution and UV detection at 250 nm. It has been suggested that the addition of silver nitrate to the extraction mixture prevents the extraction of DTT and thereby the formation of vitamin K-derived products on the HPLC column (Thijssen, 1987). To increase the accuracy of the assay, vitamin E acetate (Thijssen, Janssen, & Drijt-Reijnders, 1986) or vitamin K₁₂₅ (Chu, Huang, Williams, & Stafford, 2006) was included in the extraction solvent as the internal standard. Over the past three decades, HPLC-based K-vitamin separation and detection has been the standard *in vitro* activity assay of VKOR for its functional study. Fig. 2 shows a typical chromatogram of the activity assay for VKOR's reduction of KO to vitamin K. In addition to directly assaying VKOR's ability to reduce KO or vitamin K, indirect approaches, coupling VKOR activity with VKD carboxylation, have

also been used for functional studies of VKOR (refer to Section 1.3; Johan et al., 1987; Wallin & Martin, 1987).

These *in vitro* studies have significantly contributed to our understanding of KO reduction, VKOR gene identification, VKOR active site identification, and recombinant VKOR purification and characterization. However, they have been unable to explain the correlation between VKOR mutations and their clinical phenotypes (Hodroge et al., 2012). Additionally, the use of DTT in these assays makes it impossible to explore the role of the physiological reductant for VKOR active site regeneration. To address these questions, an alternative activity assay to study VKOR function in the native milieu is required.

1.2 Function Study of GGCX by *In Vitro* Activity Assay

GCX is a dual-function enzyme that accomplishes VKD carboxylation. During the process of carboxylation, the γ -hydrogen of the Glu residue of the substrate is abstracted, then CO_2 is added. Simultaneously, GGCX oxidizes KH_2 to KO to provide the energy required for the carboxylation (Larson, Friedman, & Suttie, 1981) (Fig. 1). The enzymatic activity of GGCX was first discovered in the 1970s (Girardot, Delaney, & Johnson, 1974; Shah & Suttie, 1974). It was found that radioactive $^{14}\text{CO}_2$ was incorporated into prothrombin in rats, and that the amount of $^{14}\text{CO}_2$ incorporation was increased three-to fourfold by the administration of vitamin K (Girardot et al., 1974). These authors concluded that VKD “completion” of prothrombin is actually carboxylation of Glu residues of the prothrombin precursor. This has been further confirmed by an *in vitro* assay using the postmitochondrial supernatant of rat hepatocytes (Shah & Suttie, 1974). Structural studies of bovine prothrombin have established that the 10 Gla residues are located at the protein’s N-terminal region (amino acid residues 1–42), now known as the Gla domain (Magnusson, Sottrup-Jensen, Petersen, Morris, & Dell, 1974). Stenflo et al. identified the structure of the Gla residues, and showed that Glu residues in prothrombin are modified by replacement of one hydrogen on the γ -carbon atom by a carboxyl group (Stenflo et al., 1974). These studies laid the foundation for the functional study of GGCX.

The functional studies of GGCX were originally based on determining the incorporation of radioactive $^{14}\text{CO}_2$ into the endogenous microsomal protein precursor substrates (Esmon, Sadowski, & Suttie, 1975). Detergent-solubilized rat liver microsome was shown to be an efficient system for GGCX function studies *in vitro* (Mack et al., 1976). As crude microsomal extracts from normal animals contain only a very small amount of endogenous protein substrates (mainly the precursors of prothrombin and factor X), treatment of animals with warfarin or feeding of animals on vitamin K-deficient diets was shown to be beneficial in obtaining substrate-enriched microsomal preparations for GGCX function studies (Vermeer, Soute, De Metz, & Hemker, 1982). Using this assay, it has been shown that the active form of vitamin K for GGCX is KH_2 , and that the enzymatic activity of GGCX is dependent on oxygen and bicarbonate (Sadowski, Esmon, & Suttie, 1976). Jones et al. reported that CO_2 is the active specie participating in VKD carboxylation of Glu residues in the protein substrates (Jones, Gardner, Cooper, & Olson, 1977). VKD carboxylation has been associated with the oxidation of KH_2 to KO (Sadowski, Schnoes, & Suttie, 1977), and

a detailed stoichiometry study of Glu carboxylation and KO formation has confirmed that the two reactions are actually carried out by the same enzyme (Larson et al., 1981).

Nowadays, the *in vitro* functional studies of GGCX were mainly carried out using artificial peptide substrates. One artificial substrate that has been used for GGCX function studies is the pentapeptide FLEEV (corresponding to residues 5–9), a peptide based on the bovine prothrombin protein sequence. It appears that the conditions for FLEEV carboxylation are identical with those for prothrombin carboxylation (Suttie & Hageman, 1976). By comparing the carboxylation of endogenous protein precursors and a variety of short peptide substrates, these authors showed that synthetic peptide substrates are carboxylated less effectively than endogenous protein substrates (Rich, Lehrman, Kawai, Goodman, & Suttie, 1981; Suttie, Lehrman, Geweke, Hageman, & Rich, 1979). In addition, a fivefold higher vitamin K concentration is required for half-maximal carboxylation of the peptide substrates than the protein substrates. GGCX carboxylates only glutamic acid residues, not aspartic acid and other related residues. It appears that hydrophobic residue around sequence EE is associated with enhanced substrate activity. As the addition of detergent to intact microsomes resulted in a 10-to 20-fold stimulation in carboxylation of a peptide substrate, it was proposed that the active site of the GGCX is accessible only from the lumen of the microsomal membrane (Carlisle & Suttie, 1980).

The most popular substrate used for GGCX functional study *in vitro* is FLEEL (corresponding to residues 5–9 of rat prothrombin protein sequence). However, FLEEL is a poor substrate analog for GGCX for the following reasons: (1) of the two glutamic acids, only the first glutamic acid of FLEEL is carboxylated during *in vitro* activity assay (Burgess, Esnouf, Rose, & Offord, 1983; Decottignies-Le Marechal, Rikong-Aide, Azerad, & Gaudry, 1979); (2) only small amounts of substrate are converted to product (Soute, Ulrich, & Vermeer, 1987); and (3) FLEEL binds poorly to GGCX, with a K_m of ~ 4 mM (Suttie et al., 1979). Knowing that GGCX interacts with clotting-factor precursors, mainly through an 18-amino acid propeptide (Jorgensen et al., 1987; Pan & Price, 1985), and since propeptide significantly increases small peptide substrate carboxylation (Knobloch & Suttie, 1987), Ulrich et al. used a 28-residue peptide, based on residues –18 to +10 in prothrombin (proPT28), as the peptide substrate of GGCX (Ulrich, Furie, Jacobs, Vermeer, & Furie, 1988). This 28-residue peptide is efficiently carboxylated, with a K_m that is three orders of magnitude lower than FLEELs. In addition to the 28-residue peptide, a 59-residue peptide, containing the propeptide sequence and the whole Gla domain (residues –18 to 41) of factor IX (proFIX59), was also proved to be an efficient substrate for *in vitro* carboxylation study; the apparent K_m (0.55 μ M) of proFIX59 is ~ five times lower than that of proPT28 (Wu, Soute, Vermeer, & Stafford, 1990). Finally, decarboxylated BGP and MGP are also good substrates for GGCX function studies (Engelke, Hale, Suttie, & Price, 1991; Vermeer, Soute, Hendrix, & de Boer-van den Berg, 1984).

GGCX activity assays are mainly based on the incorporation of $^{14}\text{CO}_2$ into VKD protein precursors or synthetic peptides. The $^{14}\text{CO}_2$ incorporation into the γ -[^{14}C]carboxyglutamate product is then determined in either the trichloroacetic acid (TCA) insoluble fractions (for the protein substrates) or the TCA soluble fractions (for the peptide substrates). After terminating the reaction with TCA, investigators must remove the unreacted $^{14}\text{CO}_2$ by

manipulating the assay tubes containing volatile $^{14}\text{CO}_2$ in potentially unsafe circumstances: centrifugation (Esmon et al., 1975) or pipetting and vigorous bubbling of the samples with carbon dioxide (Suttie & Hageman, 1976). In order to optimize safety, accuracy, and simplicity, the method for determining $^{14}\text{CO}_2$ incorporation into the peptide substrates was modified by instead boiling the TCA-treated samples in an exhaust hood (Romiti & Kappel, 1985; Ulrich et al., 1988). Recently, Kaesler et al. developed a nonradioactive assay for GGCX function study that used a fluorescein isothiocyanate (FITC)-labeled hexapeptide (FLEELK-FITC) that can be readily separated and detected in its unmodified and γ -glutamyl carboxylated form by reversed-phase HPLC with fluorescence detection (Kaesler et al., 2012).

In addition to the enzymatic catalysis assays used for GGCX function studies, Presnell et al. described a fluorescence assay for studying the interaction between GGCX and propeptide (Presnell, Tripathy, Lentz, Jin, & Stafford, 2001). This assay allows measurement of the number of propeptide-binding sites, the equilibrium dissociation constant for the propeptide–GGCX interaction, and the off-rate of a propeptide from the enzyme. These authors show that the off-rate for the FIX propeptide is 3000-fold slower than the rate of carboxylation, a difference that may explain how GGCX can carry out multiple carboxylations of a substrate during the same binding event. In order to stabilize GGCX and provide a native-like environment for its function study, GGCX was recently incorporated as single molecules into nanodiscs (Fig. 3). The assembled GGCX nanodiscs are structurally stable and catalytically active (Hebling et al., 2010). Hydrogen/deuterium exchange mass spectrometry was used to characterize specific regions of nanodisc-incorporated GGCX that exhibited structural rearrangements upon binding to propeptides. Results from these studies showed that the propeptide-binding region is located in the C-terminal of GGCX (Hebling et al., 2010; Parker et al., 2014); this is consistent with regions of propeptide binding previously identified by peptide-based affinity labeling and enzyme kinetic studies.

Most of our knowledge about GGCX's function has been obtained from these *in vitro* studies carried out under artificial conditions. Thus, we lack understanding of how GGCX carboxylates the natural VKD protein substrates in the native milieu. For example, it is not clear why some of the GGCX mutations found in patients cause carboxylation deficiencies in coagulation factors (which result in bleeding disorders), while others cause undercarboxylation of extrahepatic VKD proteins such as MGP (which results in vascular calcification). Therefore, it is essential to develop a better activity assay to explore GGCX function in its native environment in order to clarify the complex mechanism of this multisubstrate enzyme.

1.3 Function Study of VKR by *In Vitro* Activity Assay

The enzyme that reduces vitamin K to KH_2 for VKD carboxylation is VKR. It has been proposed that *in vitro*, vitamin K can be reduced to KH_2 via two pathways: a DTT-supported warfarin-sensitive pathway, accomplished by VKOR, and an NAD(P)H-dependent warfarin-resistant pathway, catalyzed by NAD(P)H quinone oxidoreductase 1 (NQO1) (Wallin & Martin, 1987; Whitlon et al., 1978). However, there is no *in vivo* experimental evidence to support this dual-pathway hypothesis. When VKOR is inactivated by warfarin or knocked

out by gene targeting in HEK293 cells, those cells can still efficiently reduce vitamin K to support VKD carboxylation (Tie, Jin, Straight, & Stafford, 2011; Tie, Jin, Tie, & Stafford, 2013). Additionally, a phenotype of VKOR-deficient mice that died due to extensive intracerebral hemorrhage could be rescued by the oral administration of vitamin K (Spohn et al., 2009). These *in vivo* studies suggest that VKOR is not the key player for vitamin K reduction in physiological conditions. NQO1 is a ubiquitous dicoumarol-sensitive flavoprotein that can catalyze the two-electron reduction of several quinones (including vitamin K) to hydroquinones (Fasco & Principe, 1982). It has long been proposed as the warfarin-resistant enzyme for vitamin K reduction (Ernster, Lind, & Rase, 1972); however, NQO1-knockout mice do not have bleeding problems (Gong, Gutala, & Jaiswal, 2008), and NQO1-deficient mice survived at the same frequency as wild-type mice when poisoned with warfarin (Ingram, Turbyfill, Bledsoe, Jaiswal, & Stafford, 2013); these *in vivo* results suggest that NQO1 does not play a major role in the production of KH_2 to support VKD carboxylation.

Despite decades of research into VKR, its identity is still unknown. However, there are several lines of evidence that demonstrate the existence and the importance of VKR. Most notably, patients that are overdosed with warfarin can be rescued by large doses of vitamin K through the action of a warfarin-resistant enzyme, designated the “antidotal enzyme,” that reduces vitamin K. The antidotal effect of vitamin K was first discovered in 1966 (O'Reilly & Aggeler, 1966) and was recently demonstrated in the VKOR-knockout mouse (Spohn et al., 2009) and in a cell-based vitamin K cycle enzyme study (Tie et al., 2011). In addition, we have observed, in AV12 cells derived from Syrian golden hamsters, a warfarin-sensitive VKR activity that is distinct from VKOR (Tie et al., 2011). A detailed understanding of the characteristics of both the warfarin-resistant and warfarin-sensitive VKRs will not only help us to understand the mechanism of vitamin K reduction, but will also be important for regulating blood coagulation and other mechanisms of physiological homeostasis.

As in the study of VKOR, function studies of VKR can be performed by directly measuring the reduction of vitamin K to KH_2 using a HPLC-based assay (Fasco & Principe, 1980). As KH_2 is unstable and can easily be oxidized back to vitamin K during extraction and isolation, KH_2 was stabilized in one of two ways: by including butylated hydroxytoluene in the vitamin K extraction buffer (Thijssen, Soute, Vervoort, & Claessens, 2004) or by performing the reaction and subsequent vitamin K isolation in sealed vials under an N_2 environment (Rishavy et al., 2013). The ability of purified recombinant VKOR to reduce vitamin K to KH_2 has been studied by directly measuring KH_2 production (Chu et al., 2006). Results show that VKOR can reduce vitamin K using the same active site cysteine residues that it uses to reduce KO, and that both activities are sensitive to warfarin inhibition (Jin, Tie, & Stafford, 2007). Rishavy et al. reported that VKOR exists as a dimer, with one molecule that reduces KO and another that reduces vitamin K (Rishavy et al., 2013). Furthermore, it has been shown that VKOR-like enzyme (VKORL, a paralog enzyme of VKOR) can also reduce vitamin K to KH_2 (Westhofen et al., 2011). Despite the fact that VKOR can function as VKR *in vitro*, it appears that in physiological conditions VKOR is not the main contributor for vitamin K reduction (Tie et al., 2011).

In addition to directly measuring KH_2 production, VKR function studies have also been carried out using an indirect method that coupled vitamin K reduction with VKD carboxylation and determined the VKR-supported incorporation of $^{14}\text{CO}_2$ either into the endogenous VKD protein substrate (Wallin, Gebhardt, & Prydz, 1978) or into the synthetic peptide substrates (Johan et al., 1987; Wallin & Martin, 1987). Microsomes prepared from animal liver were used to mimic the *in vivo* vitamin K metabolism *in vitro*. Using this coupled *in vitro* activity assay system, Wallin et al. have confirmed the existence of both the warfarin-sensitive and warfarin-resistant pathways for vitamin K reduction (Wallin & Martin, 1987). In addition, these authors have shown that the reduction of vitamin K is the rate-limiting step in VKD carboxylation (Wallin, Sane, & Hutson, 2002), which has been further confirmed by studying VKD coagulation factor carboxylation in mammalian cells (Hallgren, Qian, Yakubenko, Runge, & Berkner, 2006; Sun, Jin, Camire, & Stafford, 2005; Wajih et al., 2005). Recently, Rishavy et al. reported a similar assay system that coexpressed VKOR and GGCX in insect cells lacking endogenous VKD carboxylation components (Rishavy et al., 2013). VKR function was studied using the coupled activity assay system from intact microsomes. This *in vitro* coupling-assay approach appears reasonable for studying vitamin K metabolism and VKD carboxylation; however, the enzymes of the vitamin K cycle have very different lipid and/or detergent requirements for their activities (Carlisle & Suttie, 1980; Hildebrandt, Preusch, Patterson, & Suttie, 1984), which makes it difficult to study the interactions and activities of these enzymes together *in vitro*.

As for VKOR and GGCX, an activity assay for VKR in physiological conditions would help to understand the role of this enzyme in the complex vitamin K cycle. However, because VKR's identity is unknown, establishing such an assay is even more challenging. We hope that the recently emerged genome-scale CRISPR-Cas9 knockout screening will accelerate the identification of VKR. Detailed insights into the structure–function relationships of VKR will not only extend our understanding of the mechanism of vitamin K reduction, but will also help us to develop new ways of regulating coagulation and treating thrombosis.

2. CELL-BASED ASSAY FOR FUNCTIONAL STUDY OF VITAMIN K CYCLE ENZYMES

2.1 Rationale

To better understand VKD carboxylation in the native milieu, we developed a cell-based reporter assay system for functional study of the vitamin K cycle enzymes (Tie et al., 2011). In this system, we expressed a chimeric VKD reporter protein in mammalian cells; its carboxylation status is easily measured by enzyme-linked immunosorbent assay (ELISA). The efficiency of carboxylation of the reporter protein was used to determine the functionality of vitamin K cycle enzymes. This approach has the advantage of assessing the functionality of the vitamin K cycle enzymes in an environment that requires the enzymes to interact with their physiologic partners and/or other components of the vitamin K cycle. We used two mammalian cell lines, HEK293 and AV12, which have all the necessary components for VKD carboxylation. The HEK293 cells can efficiently carboxylate VKD proteins and have been commonly used to produce commercial therapeutic recombinant coagulation factors (Dumont, Euwart, Mei, Estes, & Kshirsagar, 2016). The AV12 cells, on

the other hand, are less efficient in the carboxylation of VKD proteins (Yan et al., 1990), which could suggest that these cells use different vitamin K pathways for making VKD proteins. By expressing an appropriate VKD reporter protein in these two cell lines, and by manipulating factors that affect VKD carboxylation, we hoped to determine the contributions of the various enzymes in vitamin K cycle.

In the HEK293 and AV12 cells, we stably expressed a chimeric VKD reporter protein, protein C with its Gla domain exchanged with that of factor IX (FIXgla-PC). The functionality of the vitamin K cycle enzymes is evaluated by culturing the reporter cells under various conditions and by using ELISA to measure the amount of carboxylated reporter protein secreted in the cell culture medium (Fig. 4). The rationale for using FIXgla-PC as the reporter protein is that the uncarboxylated protein C undergoes selective intracellular degradation through a quality control mechanism in the ER (Tokunaga, Takeuchi, Omura, Arvan, & Koide, 2000; Tokunaga, Wakabayashi, & Koide, 1995) that decreases the background secretion of reporter protein; FIXgla enables the ready quantitative detection of the carboxylated reporter protein by a calcium-dependent conformation-specific monoclonal antibody (α -FIXgla mAb) that recognizes only the fully carboxylated FIXgla (Feuerstein et al., 1999). Additionally, using the sandwich-based ELISA (Fig. 4C) to detect the carboxylated *chimeric* reporter protein eliminates the interference of the endogenous protein C or FIX in the cell culture medium. Furthermore, ELISA's detection of the reporter protein can be easily adapted with high-throughput screening of potential inhibitors of the vitamin K cycle enzymes.

Recently, similar cell-based assays have been performed using different reporter proteins (Fregin et al., 2013; Haque, McDonald, Kulman, & Rettie, 2014). Haque et al. engineered an HEK293-derived reporter cell line (HEK293-C3) that expresses a chimeric reporter protein, F9CH, which is a VKD integral membrane protein (human proline-rich Gla protein 2) with its Gla domain replaced by FIXgla (Haque et al., 2014). This chimeric reporter protein is present mainly on the cell surface, with its FIXgla domain exposed outside the cell. Carboxylated reporter proteins were detected by staining the cells with fluorescein-labeled α -FIXgla mAb, which only recognizes carboxylated FIXgla and can be quantified using flow cytometric detection. Instead of using chimeric proteins as reporters, Fregin et al. used full-length FIX as the reporter protein in HEK293T cells; they used the activity of secreted FIX in the cell culture medium to evaluate the efficiency of VKD carboxylation (Fregin et al., 2013). It should be noted that, unlike the other two assays, Fregin's assay requires transient expression of the reporter protein in each individual sample, which can create larger variations in reporter protein expression between samples.

Unlike the traditional *in vitro* activity assays, these cell-based assays provide a platform for the functional study of enzymes in the vitamin K cycle within a cellular milieu, where all the endogenous components required for VKD carboxylation are present. Therefore, results obtained from these assays for vitamin K cycle enzymes are expected to reflect their functions in physiological conditions.

2.2 Establishment of the FIXgla-PC Reporter Cell Lines

To construct the FIXgla-PC chimeric reporter protein, the cDNA encoding human protein C was amplified by PCR using pCMV-SPORT6-protein C (Open Biosystems, Huntsville, AL) as a template. A *BstBI* (TTCGAA) restriction site was introduced at the end of protein C's propeptide (amino acid residues -4I and -3R) using codon degeneracy. The Gla domain of protein C (residues 1–46) was removed by restriction digestion using *BstBI* and *BstEII* (a unique restriction site at the end of protein C's Gla domain) and replaced with the PCR fragment encoding FIX's Gla domain flanked by the same restriction sites. This chimeric FIXgla-PC fusion was subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA) using the *XbaI* site to generate the reporter protein expression vector pcDNA3.1-FIXgla-PC.

The FIXgla-PC reporter protein was stably expressed in HEK293 or AV12 cells. Cells were transfected with pcDNA3.1-FIXgla-PC plasmid DNA using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Twenty-four hours posttransfection, cells were detached and diluted at 1:10 with fresh complete growth medium (DMEM/F-12 supplemented with 10% FBS and 1 × Penicillin/Streptomycin) and plated into a 10-cm dish. The following day, selective medium with 300 µg/mL of hygromycin was added and cells were cultured at 37°C in the CO₂ incubator until cells that did not have the plasmid DNA incorporated into their genomic DNA were killed by hygromycin. Surviving colonies were manually picked and amplified in a 24-well plate in order to screen for stable expression of the reporter protein. When wells were ~ 70% confluent, 11 µM vitamin K was included in the complete growth medium and incubated for 48 h. Cell culture medium was collected and used directly for quantification of the secreted carboxylated FIXgla-PC by ELISA. The colony with the highest FIXgla-PC production was selected as the stable cell line to be used for reporter protein expression.

To further purify the manually picked colonies, the colony with the highest FIXgla-PC expression was further screened using ClonePix FL technology (CDI Bioscience, Madison, WI). Cells were partially immobilized by plating in the semisolid medium to form single colonies. After treatment with vitamin K, carboxylated reporter protein FIXgla-PC that was secreted from the colonies, which was trapped in the vicinity of the colonies by the semisolid medium, was probed by fluorescein-labeled α-FIXgla mAb. Colonies that highly express FIXgla-PC were isolated using ClonePix FL, expanded, and characterized by ELISA. Characterization of the selected colonies was performed by culturing the cells under different conditions. As expected, secretion of carboxylated reporter protein increased with the increasing concentrations of vitamin K in the cell culture medium. When these cells were fed with KO, carboxylation of the reporter protein was significantly inhibited by warfarin, a VKOR inhibitor (Fig. 5).

2.3 ELISA-Based Evaluation of the Reporter Protein Carboxylation

In this cell-based assay, the functions of vitamin K cycle enzymes were evaluated by determining the carboxylated FIXgla-PC in the cell culture medium directly, using ELISA (Tie et al., 2011). Reporter cells were seeded at ~ 60% confluence in a 24-well plate. The following day, the cell culture medium was changed to complete growth medium, which was

supplemented with either the desired substrates (such as KO or vitamin K) or with an inhibitor (such as warfarin). The cell culture medium was collected and used directly for quantification of the secreted carboxylated FIXgla-PC by ELISA after 48 h incubation.

ELISA plates were coated overnight at 4°C with 100 µL/well of α-FIXgla mAb (GMA-001, Green Mountain Antibodies, Burlington, VT). The concentration of the coating antibody was 2 µg/mL in 50 mM carbonate buffer (pH 9.6). After being washed five times with TBS-T washing buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20), the plate was blocked with 0.2% bovine serum albumin in TBS-T washing buffer for 2 h at room temperature. Samples and protein standards (0.12–250 ng/mL) containing 3 mM CaCl₂ were added at 100 µL/well and incubated for 2 h at room temperature. After being washed with TBS-T washing buffer containing 3 mM CaCl₂, 100 µL of 1:2500 diluted sheep antihuman protein C IgG conjugated to horseradish peroxidase (SAPC-HRP, Affinity Biologicals Inc., Ancaster, ON, Canada) was added to each well and incubated for 45 min at room temperature. As α-FIXgla mAb is calcium dependent, it was important to include 3 mM Ca²⁺ in any part of the procedure in which the antibody and the antigen were bounded. After the unbound detecting antibody was washed off, 100 µL of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; ABTS) solution (KPL, Gaithersburg, MD) was added to each well and the absorbance was determined at 405 nm with a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA).

To obtain the protein standard for ELISA, carboxylated FIXgla-PC chimeric protein was purified by affinity chromatography. Anticarboxylated FIXgla mAb was coupled to Affi-Gel 10 (Bio-Rad Laboratories Inc., Hercules, CA) according to the manufacturer's protocol. HEK293 cells stably expressing a high level of FIXgla-PC reporter protein were cultured in complete growth medium supplemented with 11 µM vitamin K. The medium was collected after 48 h of incubation. Calcium chloride is added to the collected medium, to a final concentration of 3 mM. Five hundred milliliters of the collected medium were incubated with 1.5 mL of the prepared anti-carboxylated FIXgla affinity resin overnight at 4°C with gentle rotation. The beads were spun down and packed into a 1.5 × 10 cm column. The column was washed first with buffer containing 20 mM Tris-HCl, 500 mM NaCl, and 3 mM CaCl₂, and then with 20 mM Tris-HCl, 100 mM NaCl, and 3 mM CaCl₂. Carboxylated FIXgla-PC reporter protein was eluted with 20 mM Tris-HCl, 100 mM NaCl, and 10 mM EDTA. The concentration of the eluted protein was calculated from its absorbance at 280 nm according to the extinction coefficient of $^{1\%}E_{280\text{nm}} = 14.5$ (Kisiel & Davie, 1981).

With the affinity-purified carboxylated FIXgla-PC as the standard protein, the standard curve for determining the carboxylated reporter protein with ELISA was plotted using a logit-log curve-fitting model (Plikaytis, Turner, Gheesling, & Carlone, 1991). In this model, logit OD₄₀₅ is plotted against the log standard protein-dilution scale. Where p represents a proportion, the logit of that proportion can be obtained using the equation $\text{logit}(p) = \log[p/(1-p)]$. Therefore, $\text{logit OD}_{405} = \log[\text{OD}_{405}/(1-\text{OD}_{405})]$. Fig. 6 shows a typical standard curve of a serial dilution of purified carboxylated FIXgla-PC, plotted by the logit-log curve-fitting model. The linear range for detection of the carboxylated reporter protein is between 0.24 and 125 ng/mL ($\gamma = 0.9991$).

2.4 Applications

The established cell-based assay allows one to study the vitamin K cycle enzymes in their native milieu. Two reporter cell lines can be used depending on the question being addressed. The HEK293 reporter cells have endogenous KO reductase activity that is warfarin sensitive and VKR activity that is warfarin resistant; AV12 reporter cells do not have warfarin-resistant VKR activity. By supplementing with either KO or vitamin K as the substrate, these cell lines can be used to study the function of enzymes involved in the redox cycling of vitamin K.

To study the two-step reduction of KO to KH₂ by the enzymes in the vitamin K cycle (Fig. 1), FIXgla-PC/HEK293 reporter cells were cultured with complete growth medium containing either KO or vitamin K. Similar levels of carboxylated reporter protein were secreted independently of the substrate (vitamin K or KO) fed to the cells (Tie et al., 2011), indicating that these cells can efficiently reduce KO to vitamin K and vitamin K to KH₂ to support VKD carboxylation. This allows one to differentiate the functions of the various enzymes involved in two-step reduction of vitamin K in the cycle. With KO as the substrate, FIXgla-PC carboxylation was completely inhibited by 2 μ M warfarin, which suggests that VKOR, the molecular target for warfarin, is responsible for the reduction of KO *in vivo*. On the other hand, when vitamin K was the substrate, the reporter cells produced unaffected levels of carboxylated reporter protein in the presence of warfarin, suggesting that with enough vitamin K in the medium, inactivation of VKOR by warfarin does not affect the conversion of vitamin K to KH₂. This result suggests that there must be a warfarin-resistant enzyme that reduces vitamin K to KH₂ in HEK293 cells, data that agree with the antidotal effect of high-dose supplementation with vitamin K upon patients poisoned with warfarin. This cell-based assay has also clarified that the warfarin-resistant pathway for vitamin K reduction in HEK293 cells is not accomplished by NQO1, the previously proposed antidotal enzyme. In AV12 cells, however, warfarin significantly inhibited FIXgla-PC carboxylation when vitamin K was used as the substrate (Tie et al., 2011). This suggests that vitamin K reduction in AV12 cells is carried out mainly via a warfarin-sensitive pathway.

It has been shown that, *in vitro*, VKOR can also reduce vitamin K through warfarin-sensitive pathways (Jin et al., 2007). To test the ability of VKOR to reduce vitamin K to KH₂ *in vivo*, tyrosine 139 in VKOR was mutated to phenylalanine (Y139F), which converts VKOR to a warfarin-resistant form (VKOR-Y139F). This permitted the inactivation of the endogenous VKOR by warfarin while testing the function of the exogenously expressed VKOR-Y139F. Because AV12 cells do not have warfarin-resistant enzyme(s) for vitamin K reduction, this cell line is a useful tool for studying vitamin K reduction by VKOR-Y139F. AV12 reporter cells expressing VKOR-Y139F failed to produce carboxylated FIXgla-PC when fed KO in the presence of warfarin, suggesting that VKOR has very limited ability to convert vitamin K to KH₂ *in vivo* (Tie et al., 2011).

The strategy of using VKOR-Y139F to study VKOR function in the cell-based assay allowed us to clarify the disputed function of the conserved loop cysteines in human VKOR and to explore the function of bacterial VKOR homologs (VKORHs). It has been proposed that, like VKORHs, the two conserved loop cysteines of human VKOR shuttle electrons to its active site cysteines through an intramolecular electron transfer pathway (Li et al., 2010;

Schulman et al., 2010). To test this hypothesis using the established cell-based assay, we first determined whether VKORHs could function in the mammalian vitamin K cycle to support VKD carboxylation. We expressed five VKORHs in HEK293 and AV12 reporter cell lines. Results show that *Mt*-VKORH (a VKORH from *Mycobacterium tuberculosis*) can efficiently support reporter-protein carboxylation using either KO or vitamin K as a substrate. Mutating both loop cysteines to alanine in *Mt*-VKORH or in human VKOR had only a minor effect on the activity of reporter-protein carboxylation, suggesting that these loop cysteines are not required for vitamin K cycling (Tie, Jin, & Stafford, 2012b). However, the loop cysteines of *Mt*-VKORH are essential for its activity in disulfide bond formation during protein folding in *Escherichia coli* (Wang, Dutton, Beckwith, & Boyd, 2011). These results together suggest that, in their respective native environments, human VKOR and VKORHs employ different mechanisms for active site regeneration. However, when *Mt*-VKORH is in the mammalian cell system, it employs a mechanism similar to that of human VKOR.

Using the reporter cell line HEK293-C3, Haque et al. have shown that *S*-warfarin (Fig. 7) is ~ 10 times more potent than *R*-warfarin for inhibiting VKD carboxylation (Haque et al., 2014), which is consistent with the potencies of warfarin enantiomers as oral anticoagulant drugs. In addition, these authors have studied the structure–activity relationship of warfarin metabolites used for inhibition of VKD carboxylation. Results from this cell-based assay, which agree with the early *in vivo* studies, show that all of the monohydroxy metabolites of warfarin (except for 10-hydroxywarfarin and warfarin alcohols) show less potency for inhibiting VKD carboxylation. Based on their results, these authors proposed that steric constraints for binding of warfarin to its target protein VKOR are greatest around the C-7 and C-8 positions, but that much more flexibility exists in the region of the VKOR active site that contacts the C-6 position (Fig. 7).

These cell-based assays allowed us to access the physiological functions of the vitamin K cycle enzymes in a way not previously possible. The strategy of creating a warfarin-resistant version of VKOR makes it possible to explore VKOR's function in the cellular environment by inactivating the endogenous VKOR with warfarin (Tie, Jin, & Stafford, 2012a; Tie et al., 2012b). However, the disadvantage of this strategy is that all the experiments must be performed in the presence of warfarin. This makes it impossible to study the warfarin resistance of the naturally occurring VKOR mutations, and it potentially compromises functional study of the wild-type enzyme. Therefore, functional study of these enzymes with a clean background requires knockout of the endogenous enzymes in the reporter cells.

3. FUNCTIONAL STUDY OF VKOR USING TALENs-MEDIATED GENE KNOCKOUT REPORTER CELLS

3.1 Rationale

VKOR is the enzyme that reduces KO in the vitamin K cycle (Fig. 1). It is the target of warfarin, the most widely used oral anticoagulant for prevention and treatment of thrombosis. However, achieving the desired anti-coagulation is challenging because of warfarin's narrow therapeutic index and the broad individual variability of dosing

requirements. With the discovery of the gene that encoding VKOR (Li et al., 2004; Rost et al., 2004), studies suggest that genetic variations in VKOR significantly contribute to warfarin dosage variability (Lee & Klein, 2013; Zineh, Pacanowski, & Woodcock, 2013). It is estimated that ~ 30% of patients receiving warfarin would benefit from VKOR pharmacogenetics modeling at the beginning of warfarin therapy (Eriksson & Wadelius, 2012). VKOR pharmacogenetics is thought to be clinically useful enough that, in 2010, the US Food and Drug Administration revised the warfarin product labels to include VKOR genotypes in the warfarin dose recommendations (Administration & U. S. F. a. D., 2010).

Missense mutations that have been identified in the VKOR coding regions of warfarin-resistant patients are believed to produce VKOR molecules that are more resistant to warfarin inhibition, thus requiring higher therapeutic warfarin doses. The traditional DTT-driven VKOR activity assay has been used to evaluate the effect of these mutations on VKOR activity and its warfarin resistance. However, except for the mutation at residue 139 in human VKOR, the results from *in vitro* studies of other VKOR mutations cannot explain the warfarin-resistant phenotype (Pelz et al., 2005; Rost et al., 2004). Recently, Hodroge et al. systematically studied all the naturally occurring VKOR mutants identified in patients requiring high dosages of oral anticoagulants (Hodroge et al., 2012). They expressed these VKOR mutants in *Pichia pastoris* and prepared VKOR-containing microsomes for functional studies using an *in vitro* DTT-driven activity assay. This study showed that of the 25 VKOR mutants, only 6 had increased resistance toward anticoagulant drugs; 10 had essentially no *in vitro* activity, which makes it difficult to explain how these VKOR variants function *in vivo*. These discrepancies presumably arise from the *in vitro* activity assay itself, which was performed in artificial conditions using the nonphysiological reductant DTT.

The cell-based assay in Section 2 has the advantage of studying the function of VKOR in its native milieu with all the required endogenous components. The disadvantage, however, is that the endogenous enzymes provide a high background. Therefore, it is not appropriate to directly use this cell-based assay to evaluate the functional consequences of VKOR mutations. To overcome this problem, the endogenous VKOR gene was knocked out in the reporter cells using transcription activator-like effector nucleases (TALENs)-mediated genome editing (Sanjana et al., 2012). Targeted gene knockout is achieved through the DNA repair machinery's action upon the chromosomal DNA double-strand breaks (DSBs) caused by TALENs in the reporter cells (Fig. 8A). TALENs consist of a pair of DNA cleavage domains of endonuclease *FokI* that have been fused to customized DNA-binding domains that recognize a specific region of the targeted gene sequence. When TALENs bind to the target sequences, the fused *FokI* are brought close enough to form a homodimer in the target spacer region. This homodimer of *FokI* is the active form, which will create DSBs in the genomic DNA. The repair of the DSBs in mammalian cells by nonhomologous end-joining (NHEJ) pathways introduces mutations via random insertions or deletions (indel mutations; Fig. 8B). This often produces frameshift mutations that ablate the targeted gene function.

Based on loss-of-function screening and characterization of TALENs-mediated gene targeting in the above reporter cells, both VKOR and VKORL must be knocked out to eliminate the ability of HEK293 cells using KO as substrate to support VKD carboxylation (Tie et al., 2013). Reporter cells with both VKOR and VKORL knocked out (double-gene

knockout, or DGKO) provide an ideal tool for VKOR function study with a clean background.

3.2 Establishment of the TALENs-Mediated VKOR/VKORL Knockout Reporter Cell Line

To knock out the endogenous VKOR and VKORL genes in HEK293 cells, TALENs recognition sites that target exon 1 (near the start codon region of human VKOR and VKORL) in the genomic DNA sequences were designed using TALE-NT 2.0 software (Doyle et al., 2012). Both the left and the right TALENs were designed to target a 20-bp sequence, with 17-bp spacing for VKOR and 15-bp spacing for VKORL (Fig. 9). The 18-mer DNA-binding arrays of TALEN were engineered using TALE Toolbox, as described previously (Sanjana et al., 2012), with minor modification. Instead of generating 72 monomers (18 monomers for each TALE DNA-binding domain), 40 monomers were created using PCR to avoid monomer repeats. PCR products for making TALENs DNA-binding arrays were purified using gel purification. The assembled TALENs were cloned into the corresponding pTALEN_v2 vectors provided in the TALE Toolbox kit. The integrity of each assembled TALEN was verified by sequence analysis.

The reporter cell line of FIXgla-PC/HEK293 was used for TALENs-mediated VKOR gene knockout. Plasmid DNA containing the left and right TALENs targeting VKOR was transiently expressed in FIXgla-PC/HEK293 cells. Forty-eight hours posttransfection, cells were treated with trypsin, filtered with a 30- μ m filter (Sysmex Partec GmbH, Gorlitz, Germany), and diluted with complete growth medium to obtain single-cell suspensions. Diluted cells were seeded into 96-well plates for functional screening of the gene knockout. Alternatively, single-cell suspensions of the transfected cells were plated into 150-mm dishes. Single colonies were manually picked for functional screening.

VKOR is the enzyme responsible for reducing KO to vitamin K, while HEK293 cells possess an “antidotal” enzyme that can reduce vitamin K to KH_2 . We reasoned that HEK293 cells with the VKOR gene knockout would abolish only the KO reductase activity but leave the K reductase activity intact (Fig. 1). Therefore, functional screening of VKOR knockout cell colonies was performed by first measuring the efficiency of reporter-protein carboxylation when the cell colonies were fed with 5 μM KO for 48 h. Carboxylated FIXgla-PC in the cell culture medium was measured by ELISA, as described in Section 2.3. Cell colonies with significantly reduced carboxylation of reporter protein were then fed with 11 μM vitamin K for 24 h to test whether their K reductase activity was maintained. The ratio of carboxylated FIXgla-PC produced in the KO-supplemented medium and the vitamin K-supplemented medium was used as a criterion to identify candidate cell colonies for VKOR gene knockout. To ensure that the knockout cell was clonal, functional screening was repeated as described earlier.

To verify that the loss of KO reductase activity was due to TALENs-mediated genome editing of the VKOR gene, genomic DNA was extracted from the selected VKOR knockout cells using QuickExtract DNA extraction solution (Epicentre, Madison, WI). The TALENs-targeted region in the genomic DNA was amplified by PCR using 5'-GCTTCACTAGTCCCGGCATTCTTCGCTG-3' as the forward primer and 5'-CCAGCACTGTCTGGTCCCTTGCTCGCAC-3' as the reverse primer. PCR products

were cloned into the TOPO TA cloning vector (Invitrogen, Carlsbad, CA) for sequence analysis. To verify TALENs-mediated editing of the VKOR gene at the mRNA level, total RNA from VKOR knockout cells was extracted using Trizol. RT-PCR was performed using poly T18 and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). The TALENs-targeted region was amplified by PCR, and the PCR product was cloned into the TOPO TA cloning vector for sequence analysis.

It is worth noting that VKOR knockout cells regained KO reductase activity after culturing for several generations. It appears that this regained activity is from VKORL (Tie et al., 2013), a paralog enzyme of VKOR that shares ~ 50% sequence identity with VKOR (Rost et al., 2004). Therefore, we designed TALENs pairs targeting the VKORL gene in the VKOR knockout cells. After loss-of-function screening, genomic DNA was prepared from the candidate knockout cell colonies and the targeted region was amplified by PCR using 5'-CCTTGCAATGTGCTAGTTAGACCGCT CC-3' as the forward primer and 5'-CACCAGGAAGACAGTGGGAAA GGCAACC-3' as the reverse primer. VKORL gene knockout has been confirmed by sequencing analysis of the target region. Cells with both VKOR and VKORL knocked out (DGKO) no longer support reporter-protein carboxylation using KO as the substrate (Fig. 10). However, exogenous expression of VKOR or VKORL significantly increases the production of carboxylated reporter protein, suggesting that the DGKO reporter cell line is a useful tool for VKOR function study with a clean background.

3.3 Functional Study of VKOR Using the DGKO Reporter Cells

Functional study of VKOR is based on the ability of VKOR or its mutants to reduce KO to vitamin K to support the carboxylation of the reporter protein in the DGKO reporter cells. VKOR or VKOR mutant is transiently expressed in the DGKO cells, and the KO reductase activity is evaluated by measuring the level of carboxylated reporter protein in the cell culture medium when cells fed with KO. Mammalian expression vector pBudCE4.1 (Invitrogen, Carlsbad, CA), designed for simultaneous expression of two genes in mammalian cells, was used as the basic cloning vector. The cDNA of *Metridia* luciferase was cloned into one of the multicloning sites of pBudCE4.1 (the resulting plasmid is named as pBudCE4.1-Met.Luc) and the cDNA of VKOR or its mutant was cloned into the other multicloning site. *Metridia* luciferase, coexpressed with VKOR or its variants by the same vector controlled by different promoters, was used as the internal control for normalizing the transient transfection efficiency.

For transient transfection, the DGKO cells were seeded, 1 day before transfection, into a 24-well plate in complete growth medium, so that the cells were approximately 60% confluent at the time of transfection. The plasmid DNA of pBudCE4.1-Met.Luc containing the cDNA of VKOR or its mutants was transiently transfected into the DGKO cells using Xfect transfection reagent (Clontech Laboratories, Inc., Mountain View, CA) according to the manufacturer's instructions, with minor modifications. Triplet transfections were performed for each sample. For each well, 0.5 µg plasmid DNA was diluted with 25 µL of Xfect reaction buffer. After adding 0.15 µL of Xfect polymer, samples were mixed by vortexing for 10 s at high speed. Right before use, the Xfect polymer was diluted 20-fold with Xfect reaction buffer and mixed by vortexing. The DNA-Xfect polymer complex solution was

incubated at room temperature for 10 min. Then the entire solution was added dropwise to one well containing 250 μ L complete growth medium. The plate was gently rocked back and forth to mix and incubated at 37°C with 5% CO₂ atmosphere for 4 h. The transfection medium was replaced with complete growth medium containing different concentrations of test compounds, such as KO alone or KO with different concentrations of warfarin. The plate was returned back to the 37°C CO₂ incubator. Forty-eight hours posttransfection, the medium was collected to determine the concentration of carboxylated reporter protein and to measure luciferase activity for normalizing the transfection efficiency.

The concentration of carboxylated reporter protein in the cell culture medium was measured by ELISA, as described in Section 2.3. Luciferase activity was determined by injecting 90 μ L of 2 μ M coelenterazine (NanoLight Technology, Pinetop, AZ) solution (in PBS with 300 mM NaCl) to 10 μ L of cell culture medium in a 96-well Lumitrac white plate (Greiner Bio-One GmbH, Austria, Germany). Luminescence emission from the mixture was recorded using a SpectraMax L Luminescence Microplate Reader (Molecular Devices, Sunnyvale, CA) at 480 nm with an integration time of 1 s and a delay of 6 s. VKOR activity was evaluated by reporter-protein carboxylation normalized by luciferase activity for each individual sample.

If a large number of samples need to be tested, the cell-based assays introduced in this chapter can be scaled up from the 24-well plate format to a 48-well plate format. In this case, 1 day prior to transfection, reporter cells would be seeded into 48-well plates in complete growth medium. An electronic adjustable tip spacing multichannel pipette is helpful for seeding the cells evenly in 48-well plates. For transfection, 0.25 μ g plasmid DNA should be diluted with 12.5 μ L Xfect reaction buffer. After mixing with Xfect polymer and incubating at room temperature for 10 min, the entire solution should be added dropwise to one well containing 125 μ L complete growth medium. The plate should be rocked gently back and forth to mix, then incubated at 37°C with 5% CO₂ atmosphere. After 4 h, instead of replacing the transfection medium with test medium, 125 μ L of 2 $\times$ test medium should be added directly to the transfection medium. The plate should be rocked gently back and forth to mix, and it should then be returned back to the 37°C CO₂ incubator for 48 h.

3.4 Applications

The established DGKO reporter cell line, which has all the essential endogenous components for VKD carboxylation except for KO reductase, provides an invaluable tool for studying the function of VKOR in its native milieu with a clean background. It is especially useful for studying the warfarin resistance of naturally occurring VKOR mutants (Fig. 11). Our knowledge on warfarin resistance of VKOR mutants is mainly based on *in vitro* studies that were carried out under artificial conditions using DTT to provide the reducing equivalents normally provided by unknown endogenous reductants of VKOR. Therefore, the warfarin resistance of most of the VKOR mutants as determined by the *in vitro* assay is not consistent with the clinical phenotypes of patients carrying these mutations (Hodroge et al., 2012). Although the cell-based assay described in Section 2 has been successfully used for *in vivo* functional studies of VKOR, endogenous VKOR activity in HEK293 cells prevents

its application for warfarin resistance studies, especially for those VKOR mutations with marginal resistance.

Using the DGKO reporter cell line, we examined 10 naturally occurring VKOR mutants whose warfarin resistance could not be tested *in vitro* because they had essentially no *in vitro* activity (Hodroge et al., 2012). Results from the DGKO cell-based assay show that all these VKOR mutants are fully active (Tie et al., 2013). This is consistent with a recent clinical study showing that patients with warfarin-resistant VKOR mutations have normal VKD clotting factors in the absence of warfarin (Harrington, Siddiq, Allford, Shearer, & Mumford, 2011). Compared with the wild-type VKOR, five VKOR mutants showed increased warfarin resistance. These mutants, in increasing order, are: VKOR<S52L $\approx$ N77S<W59L $\approx$ L128R<W59R. Except for the L128R mutant, this order of warfarin resistance is consistent with the corresponding clinical anticoagulant dosages. However, the warfarin sensitivity of the other five VKOR mutants is not significantly different from that of the wild-type enzyme, which suggests that not all of the naturally occurring VKOR mutants found in patients requiring higher doses of warfarin are resistant to warfarin inhibition. Other factors must also play important roles in warfarin resistance in patients.

Warfarin resistance in VKOR mutants has been studied using a nonknockout cell-based assay (Fregin et al., 2013), similar to the one described in Section 2. In general, the nonknockout cell-based assay reached a conclusion similar to that reached by the DGKO cell-based study. However, in the nonknockout cell-based assay, warfarin's 50% inhibition (IC_{50}) of both wild-type VKOR and the L128R mutant occurred at 24.7 and 1226.4 nM, respectively. These values are $\sim$ 10-fold higher than those obtained from the DGKO cell-based study (3.48 and 107 nM, respectively). The higher IC_{50} in the nonknockout cell-based assay is probably caused by endogenous VKOR in the reporter cells. An additional complication is that, instead of using KO as the substrate, this nonknockout cell-based assay used vitamin K as the substrate for the VKOR function study. Therefore, the VKOR activity measured by this approach depends on the conversion of vitamin K to KO by the HEK293 cells, a process that produces carboxylated reporter proteins independent of VKOR activity.

The DGKO reporter cell line provides an ideal tool for studying VKOR function in a cellular environment. It is especially useful for studying the warfarin resistance of the naturally occurring VKOR mutations, and for clarifying VKOR's genotype and the clinical phenotypes VKOR produces. Because of the depletion of VKORL in this cell line, it is also a potential tool for studying the structure–function relationship of VKORL, whose physiological role is still unknown.

4. FUNCTIONAL STUDY OF GGCX USING CRISPR-CAS9-MEDIATED GENE KNOCKOUT REPORTER CELLS

4.1 Rationale

The enzyme that accomplishes carboxylation is GGCX. It is responsible for carboxylation of VKD proteins to their functional forms. GGCX recognizes its protein substrate through a relatively tight binding to the propeptide of the VKD proteins, which tethers the substrate to

the enzyme (Furie, Bouchard, & Furie, 1999). The binding of the propeptide to GGCX initiates a structural reorientation of GGCX by which the Gla domain of the protein substrate is positioned at the catalytic site of GGCX (Higgins-Gruber et al., 2010), and all 10–12 glutamic acids in the Gla domain are carboxylated during one binding event (Morris, Stevens, Wright, & Stafford, 1995). Although the apparent affinities of the VKD proteins' propeptides for GGCX vary over 100-fold from *in vitro* studies (Higgins-Gruber et al., 2010), these VKD proteins appear to be fully carboxylated in physiological conditions.

Genetic screening for GGCX variations in patients with vitamin K-related disorders has identified more than 30 naturally occurring mutations (Watzka et al., 2014). However, the correlation between these GGCX mutants and their clinical phenotypes is not clear. For example, it has been well established that mutations in the GGCX gene can result in defects of carboxylation of VKD coagulation factors causing bleeding disorders, referred to as VKCFD. The mainstay of therapy for VKCFD is the administration of vitamin K, which most often completely or partially corrects the coagulation factors defect; in some cases, however, vitamin K is ineffective (Brenner, Kuperman, Watzka, & Oldenburg, 2009). The patient's vitamin K response does not seem to be correlated with either the clinical severity of the disorder or the severity of the molecular defect. Additionally, it is not clear why some mutations cause bleeding disorders while others cause nonbleeding syndromes such as Pseudoxanthoma elasticum (PXE)-like syndrome. This lack of knowledge is caused in part by the fact that our current understanding of GGCX's function was obtained from *in vitro* experimentation under artificial conditions using the pentapeptide FLEEL as the substrate. Therefore, understanding GGCX, and being able to characterize this multiple-substrate enzyme in its native milieu, will help to clarify the clinical phenotypes and treatment of vitamin K-related disorders.

In an attempt to access GGCX function in an animal model, the endogenous GGCX gene was knocked out in mice by gene targeting (Zhu et al., 2007). However, all homozygous GGCX-deficient mice succumbed to massive intraabdominal hemorrhage shortly after birth. Recently, in another attempt to study GGCX function *in vivo*, mice with liver-specific GGCX deficiency have been created (Azuma et al., 2014); however, manipulating GGCX variants and its substrates in the mouse model appears impracticable. Because manipulation of the vitamin K metabolism pathway is not lethal for mammalian cells, and because cell-based functional study of vitamin K cycle enzymes is feasible, we decided to develop a GGCX-deficient reporter cell line using CRISPR-Cas9-mediated genome editing to address questions related to GGCX catalysis in a cellular milieu.

First, we stably expressed two structurally distinct VKD proteins (FIXgla-PT and MGP) as reporter proteins in HEK293 cells. FIXgla-PT is a chimeric protein of prothrombin with its Gla domain exchanged with that of factor IX. Like other VKD coagulation factors, prothrombin is produced and carboxylated in the liver. All the Gla residues of VKD coagulation factors, located in the N-terminal Gla domain, are preceded by the propeptide, which will be cleaved in the mature protein (Fig. 12). However, MGP, which is produced and carboxylated in extrahepatic tissues, retains its propeptide within the mature protein, and MGP's Gla residues are scattered on both sides of the propeptide. While defects of carboxylation of coagulation factors result in bleeding disorders, defects of carboxylation of

MGP are mainly associated with nonbleeding syndromes; we expect that these two structurally distinct reporter proteins may be carboxylated differently by GGCX, and that certain GGCX mutations could have different effects on these reporter proteins' carboxylation, resulting in different clinical phenotypes.

We used the genome-editing tool CRISPR-Cas9 to knock out the endogenous GGCX gene in HEK293 cells that stably express the two reporter proteins, FIXgla-PT and MGP. CRISPR-Cas9 mediates gene knockout by introducing targeted DSBs in the genomic DNA of the gene of interest (Cong et al., 2013; Mali et al., 2013). The introduction of the DSB is achieved by CRISPR-Cas9 with a specific single-guide RNA (sgRNA; Fig. 13A). The sgRNA binds to Cas9, directs it to a specific genomic locus complementary to the 20-base gRNA sequence, and induces a DSB using Cas9. Subsequent repair of the DSB via the NHEJ pathway in the cell produces indel mutations at the site of DSB. Frameshift mutations at the DSB site disrupt the gene of interest (Fig. 8B). Since GGCX is the only enzyme responsible for VKD carboxylation in the cell, knocking out of the endogenous GGCX gene will abolish reporter-protein carboxylation. Therefore, we can study GGCX function by observing how efficiently the reporter proteins are carboxylated in the GGCX-knockout cells by either the exogenously expressed GGCX or its mutants.

4.2 Establishment of the CRISPR-Cas9-Mediated GGCX Gene Knockout Reporter Cell Line

To establish a reporter cell line with two reporter proteins (a dual-reporter cell line), FIXgla-PT and MGP were stably expressed in HEK293 cells. The DNA sequence encoding chimeric reporter-protein FIXgla-PT was created by overlap PCR, as previously described (Tie et al., 2011). The replacement in prothrombin of the Gla domain with the factor IX's Gla domain allowed us to use the α -FIXgla mAb for quantitative detection of the carboxylated reporter protein. To increase reporter-protein stability, the protease cleavage sites at arginine residues 155, 271, and 320 of prothrombin were mutated to glutamine (Krishnaswamy, 2013). The cDNA of prothrombin with arginine 271 and 320 mutated to glutamine was a gift from Dr. Krishnaswamy from Children's Hospital of Philadelphia, PA. MGP, the second reporter protein, has a C-terminal HPC4 tag (EDQVDPRLIDGK) for detection purposes. The DNA sequences encoding FIXgla-PT and MGP were cloned into the mammalian dual-expression vector pVITRO1-hygro-MCS (InvivoGen, San Diego, CA). Plasmid DNA of pVITRO1-hygro-MCS containing both sequences of FIXgla-PT and MGP was transfected into HEK293 cells. Forty-eight hours posttransfection, transfected cells were treated with trypsin, diluted with complete growth medium containing 0.3 mg/mL hygromycin, and seeded into 100-mm dishes. Single colonies were picked and screened for high expression of both reporter proteins.

To knock out the endogenous GGCX gene of the above reporter cells, we used CRISPR-Cas9-mediated genome editing. First, we examined the genome-editing efficacy of six gRNA sequences optimized for GGCX targeting (Shalem et al., 2014) (Fig. 13B). Each of the six gRNA and a nontargeting gRNA (control) were cloned into the lentiCRISPR V2 vector (a gift from Feng Zhang, Addgene plasmid #52961; Shalem et al., 2014). This lentiCRISPR V2 vector allows the simultaneous expression of the sgRNA, endonuclease Cas9, and a puromycin selection marker. Lentivirus, produced by cotransfection of the

lentiCRISPR V2 sgRNA constructs and the lentivirus packaging vectors (pVSVg and psPAX2) into HEK293T cells, was used to infect our reporter cells (FIXgla-PC/HEK293, Section 2.2) for the CRISPR-Cas9-mediated GGCX gene knockout. Twenty-four hours posttransduction, cells were cultured with complete medium containing 1.5 $\mu\text{g/mL}$ puromycin; only cells transduced with a lentiCRISPR V2 sgRNA construct survived. Puromycin-resistant cells were expanded and cultured for 48 h with 11 μM vitamin K in the complete growth medium. For each gRNA, the efficiency of the genome editing in the GGCX knockout was evaluated by measuring the loss of reporter-protein carboxylation. The most efficient gRNA for GGCX targeting was used to knock out the endogenous GGCX gene in the dual-reporter cells.

Next, the selected gRNA sequence (Fig. 13B) for GGCX targeting was cloned into plasmid pX330-U6-Chimeric-BB-CBh-hSpCas9 (a gift from Feng Zhang, Addgene plasmid #42230). This plasmid allows the simultaneous expression of the selected sgRNA, for GGCX targeting, and endonuclease Cas9, for making DSBs. It is transiently transfected into the dual-reporter cells. Forty-eight hours posttransfection, cells were treated with trypsin, and a single-cell suspension was obtained as described in Section 3.2. Diluted cell suspensions were plated into 96-well plates or seeded into 150-mm dishes for loss-of-function screening of GGCX knockout.

To verify GGCX gene knockout in the candidate cell colonies, genomic DNA was prepared using QuickExtract DNA extraction solution. The gRNA-targeting region in the genomic DNA was amplified by PCR, and the PCR products were cloned into TOPO cloning vector for sequence analysis as described in Section 3.2. To further confirm the loss of endogenous GGCX expression in the GGCX-knockout cells, cell lysates were prepared and loaded to sodium dodecyl sulfate-polyacrylamide gel electrophoresis for western blot analysis. Protein bands were probed using rabbit anti-GGCX polyclonal antibody (Proteintech Group Inc., Chicago, IL). The selected positive GGCX-deficient cell colonies expressed no detectable endogenous GGCX and were unable to carboxylate the reporter proteins (Fig. 14). Expression of the exogenous GGCX in these GGCX-deficient cells restores carboxylation activity, suggesting that other components of the vitamin K cycle in the cells, as well as the reporter system, are intact.

4.3 Functional Study of GGCX Using the GGCX-Deficient Reporter Cells

Functional study of GGCX is based on the ability of GGCX or its mutants to carboxylate the two reporter proteins in the GGCX-deficient dual-reporter cells. The cDNA of GGCX or its mutants was cloned into mammalian expression vector pBudCE4.1-Met.Luc and transiently expressed in the GGCX-deficient dual-reporter cells as described in Section 3.3. Four hours posttransfection, the transfection medium was replaced by complete growth medium containing 11 μM vitamin K and incubated for 48 h. Carboxylation activity was determined by measuring the level of carboxylated reporter proteins in the cell culture medium using sandwich-based ELISA (Tie et al., 2016).

To evaluate carboxylation of FIXgla-PT, α -FIXgla mAb is used as the capture antibody, and horseradish peroxidase-conjugated sheep antihuman prothrombin (Affinity Biologicals Inc., Ancaster, Canada) is used as the detecting antibody. As described in Section 2.3, it is

essential to include 3 mM Ca^{2+} in samples and all the buffers for ELISA. To measure the carboxylation of the second reporter protein MGP, a monoclonal antibody that specifically recognizes carboxylated MGP sequence 35 through 49 (VitaK BV, Maastricht, the Netherlands) is used as the capture antibody, and biotinylated anti-HPC4 monoclonal antibody is used as the secondary antibody. Streptavidin poly-HRP (Pierce, Rockford, IL) is used to amplify the signal of MGP detection. For MGP detection, it is important to *not* include Ca^{2+} in sample and all the buffers for ELISA.

4.4 Applications

The established GGCX-deficient dual-reporter cell line has been successfully used to study GGCX function and to clarify the correlation between GGCX genotypes and their clinical phenotypes. Using this cell-based assay, we first studied GGCX mutations found in a child with severe cerebral bleeding and comorbid Keutel syndrome, a nonbleeding malady caused by functional defects of MGP (Tie et al., 2016). Clinical data showed that high doses of vitamin K administration improved the bleeding diathesis of the patient, but not the functional defect of MGP. Genetic analysis of the GGCX gene in the patient detected three missense mutations corresponding to amino acid changes of D153G, M174R, and R325Q. To study how these GGCX mutations impacted the clinical phenotypes of the patient, we measured the carboxylation activity of these mutants using the GGCX-deficient dual-reporter cell-based assay. Results showed that the R325Q mutant is almost as efficient as the wild-type enzyme for the carboxylation of both reporter proteins. In contrast, the M174R mutant carboxylates neither reporter protein in the presence of vitamin K. As the patient had a defect of VKD coagulation factors—a defect that can be ameliorated by vitamin K supplementation—these two mutations are unlikely to explain the patient's clinical phenotypes. However, while the D153G mutant has ~ 60% activity for FIXgla-PT carboxylation in the presence of high concentration of vitamin K, it displays significantly decreased MGP carboxylation. This result is consistent with the patient's clinical result, suggesting that D153G is the causative mutation for the patient's clinical phenotypes.

To gain further insights on how the clinical phenotypes respond to vitamin K administration, GGCX-deficient dual-reporter cells expressing the causative GGCX mutant D153G were treated with increasing concentrations of vitamin K. Results show that higher vitamin K concentrations recovered the FIXgla-PT carboxylation up to ~ 60% (Fig. 15A). This is consistent with the patient's clinical results, in which the VKD coagulation factor's activity reached up to 53% after supplementation with high-dose vitamin K. Additionally, the cell-based assay shows that higher concentrations of vitamin K have only a moderate effect on MGP carboxylation (Fig. 15B), a finding that again agrees with the clinical results of the patient; administration of high doses of K vitamins failed to increase the carboxylation of MGP. These results, taken together, suggest that the GGCX-deficient dual-reporter cell-based assay is a good system for the functional study of GGCX *in vivo*.

One of the caveats of using results obtained from the GGCX-deficient cell-based assay to explain clinical phenotypes is that two of the reporter proteins in this assay are carboxylated in the same type of cell, and factors that affect VKD proteins' carboxylation in physiological conditions may behave differently in different types of cells/tissues. For example, vitamin K

(phylloquinone) is the main supplement used to treat VKCFD patients to improve the carboxylation of coagulation factors. It is the most abundant form of K vitamin in the liver (Shearer & Newman, 2014), where coagulation factors are produced and carboxylated. On the other hand, administration of menaquinones, but not phylloquinone, improves MGP carboxylation and reduces both cardiovascular mortality and coronary calcification. MGP is produced by chondrocytes, vascular smooth muscle cells, endothelial cells, and fibroblasts, and it undergoes local carboxylation in the extrahepatic tissues, where menaquinones are believed to play a role in VKD carboxylation (Danziger, 2008). Menaquinone supplementation has been shown to improve extrahepatic vitamin K status, but to have no effect on thrombin generation in healthy subjects (Theuvsen et al., 2012). However, results from the GGCX-deficient cell-based assay show that both vitamin K and menaquinones can efficiently support the carboxylation of both coagulation factors and MGP by wild-type GGCX (Tie et al., 2016). The different contributions of phylloquinone and menaquinones to hepatic and extrahepatic carboxylation may therefore be caused by the way different tissue absorbs and utilizes these K vitamins.

5. CONCLUDING REMARKS

The cell-based assays introduced in this chapter provide versatile tools for the functional study of enzymes of the vitamin K cycle in a cellular milieu. With the aid of recently developed genome-editing techniques, one can knock out a specific gene of interest in the reporter cell line and study the function of the protein in the native cellular environment. We expect that these novel cell-based assays will improve our understanding of how the various vitamin K cycle components contribute to these proteins' complex mechanisms and functions. The detailed understanding of VKOR and GGCX and their naturally occurring mutants that can be achieved using these assays will lead to new ways of controlling thrombosis, will help researchers understand warfarin therapy, and will aid in the development of new anticoagulant drugs. In addition, it is becoming clear that VKD proteins control physiologic processes that contribute to other areas of health interest. Therefore, it seems likely that studies using these cell-based assays will lead to a better understanding of the mechanisms of a variety of diseases.

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Fig. 1.

Vitamin K cycle. During vitamin K-dependent (VKD) carboxylation, the glutamate (Glu) of the VKD protein is converted to gamma-carboxyglutamate (Gla) by GGCX using vitamin K hydroquinone, carbon dioxide (CO₂), and oxygen (O₂) as cofactors. Concomitant with carboxylation, vitamin K hydroquinone is oxidized to vitamin K epoxide. Vitamin K epoxide is reduced to vitamin K by VKOR. The reduction of vitamin K to vitamin K hydroquinone is carried out by the as-yet-unidentified VKR.

Fig. 2.

Chromatogram of HPLC-based VKOR *in vitro* activity assay. The VKOR *in vitro* activity assay was performed using KO as the substrate and DTT as the artificial reducing agent. The amount of vitamin K (K) production was used to evaluate VKOR activity. K vitamins in the reaction mixture were extracted by organic solvent and separated by HPLC with UV detection at 250 nm. Negative controls were reactions that had no enzyme added (W/O VKOR) or that had no DTT added (W/O DTT). Vitamin E acetate was included in the organic extraction solvent as the internal standard (STD).

Fig. 3. Schematic diagram of GGCX incorporated into nanodisc. GGCX, a five-transmembrane domain integral membrane protein, was incorporated into lipid bilayers with the help of membrane scaffold protein (MSP) to form the nanodisc which renders GGCX soluble in aqueous solution and structurally stable.

Fig. 4.

Cell-based activity assay for vitamin K cycle enzymes with ELISA detection. (A) Domain structure of reporter protein FIXgla-PC. *AP*, activation peptide of protein C; *Catalytic domain*, region containing the serine protease catalytic triad of protein C; *EGF*, region of protein C homologous to human epidermal growth factor; *FIXgla*, Gla domain of human factor IX. (B) Schematic diagram of FIXgla-PC/HEK293 reporter cell. Reporter protein FIXgla-PC is carboxylated by the endogenous vitamin K cycle enzymes of the HEK293 cell in the presence of vitamin K. The carboxylated FIXgla-PC is secreted from HEK293 cell into the cell culture medium. (C) Sandwich-based ELISA detection of the carboxylated reporter protein. Carboxylated FIXgla-PC is captured by α -FIXgla mAb (capture antibody) in the presence of Ca^{2+} and detected by HRP-conjugated sheep antihuman protein C antibody (detector antibody).

Fig. 5. Characterization of FIXgla-PC/HEK293 reporter cells with vitamin K and warfarin. FIXgla-PC/HEK293 reporter cells were cultured for 48 h with increasing concentrations of vitamin K (•) or 5 μM KO with increasing concentrations of warfarin (◆). The concentration of carboxylated FIXgla-PC in the cell culture medium was measured by ELISA.

Fig. 6. Standard curve for measuring carboxylated reporter protein FIXgla-PC by ELISA. Affinity-purified carboxylated FIXgla-PC was serially diluted and determined by ELISA. Protein concentration and absorption at 405 nm were plotted using a logit–log curve-fitting model.

Fig. 7.
Chemical structure of warfarin enantiomers.

Fig. 8. Schematic diagram of TALENs-mediated gene knockout. (A) Customized DNA-binding domain of TALE recognizing a specific genomic DNA sequence is fused to the catalytic domain of *FokI* endonuclease (TALEN). When a pair of TALENs recognize the *top* and *bottom* strands of the target sites flanking a 14–20 bp spacer, the *FokI* dimerizes and cuts the DNA in the spacer region to create the double-strand breaks (DSBs). (B) When DSBs in a gene locus are repaired by the error-prone nonhomologous end-joining (NHEJ) pathway, it leads to the introduction of variable length insertion or deletion (indel) mutations resulting in ablation of the targeted gene function.

Fig. 9. TALENs target sequences for VKOR and VKORL knockout. The *left* and *right* TALENs binding sequences for VKOR (*top*) and VKORL (*bottom*) are highlighted. The start codon for protein translation is indicated by *arrows*.

Fig. 10.

Reporter protein carboxylation in the DGKO reporter cells. DGKO reporter cells were transiently transfected with expression vector (control), VKOR, or VKORL. Transfected cells were cultured with 5 μ M KO for 48 h. The concentration of carboxylated FIXgla-PC in the cell culture medium was measured by ELISA.

Fig. 11.

Warfarin resistance study of VKOR and its Y139F mutant using the DGKO reporter cells. VKOR or its Y139F mutant was transiently expressed in the DGKO reporter cells and the transfected cells were cultured with 5 μ M KO and increasing concentrations of warfarin for 48 h. The concentration of carboxylated FIXgla-PC in the cell culture medium was measured by ELISA.

Fig. 12.

Domain structure of the reporter proteins MGP and FIXgla-PT. *Top*: Domain structure of MGP. MGP has a propeptide sequence in the mature protein. There is one Gla residue at the N-terminus and four Gla residues at the C-terminus of the propeptide sequence. *Bottom*: Domain structure of FIXgla-PT. *Catalytic domain*, region containing the serine protease catalytic triad of prothrombin; *FIXgla*, Gla domain of human factor IX; *Kringle*, regions of internal sequence homology of prothrombin; *Propeptide*, propeptide region of prothrombin.

Fig. 13. Schematic diagram of CRISPR-Cas9-mediated gene knockout and optimized gRNA sequences for GGCX targeting. (A) Cas9-sgRNA in complex with a target site in the genomic DNA. sgRNA binds and directs Cas9 to the genomic DNA target site with the appropriate PAM sequence at the 3' end to create DSBs (indicated by the *arrows*). (B) Six optimized gRNA sequences for GGCX targeting and a nontargeting gRNA sequence used as the control. The gRNA sequence used for creating GGCX-knockout cell line is highlighted.

Fig. 14.

Functional characterization of the GGCX-knockout reporter cells. (A) Immunoblotting of wild-type (lane 1) and GGCX-knockout (lane 2) HEK293 cells. GGCX was probed by rabbit anti-GGCX polyclonal antibody (Proteintech Group Inc., Chicago, IL) as the primary antibody and horseradish peroxidase-conjugated goat antirabbit antibody (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) as the secondary antibody. The endogenous GGCX band is indicated by the *asterisk*. (B) Carboxylation activity of GGCX-knockout reporter cells and the cells transfected with wild-type GGCX. GGCX-knockout reporter cells were transiently transfected with expression vector (blank) or GGCX and cultured with 11 μ M vitamin K for 48 h. The concentration of carboxylated FIXgla-PT in the cell culture medium was measured by ELISA.

Fig. 15. Vitamin K titration of GGCX and its D153G mutant using GGCX-knockout reporter cells. Carboxylation of reporter proteins FIXgla-PT (A) and MGP (B) by GGCX (●) and D153G mutant (■) with increasing concentrations of vitamin K. GGCX or its D153G mutant was transiently expressed in the GGCX-knockout reporter cells and the transfected cells were cultured with increasing concentrations of vitamin K for 48 h. The concentrations of carboxylated reporter proteins in the cell culture medium were measured by ELISA.