Development of Antibody Modulators for an Oncogenic Potassium Channel

by

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Abstract

Two-pore domain potassium (K2P) channels act to maintain cell resting membrane potential – a prerequisite for many biological processes. KCNK9, a member of K2P family, is implicated in cancer owing to its overexpression in human tumors and its ability to promote neoplastic cell survival and growth. However, KCNK9’s underlying contributions to malignancy remain elusive due to the absence of specific modulators. Here, we describe the development of novel monoclonal antibodies against the KCNK9 extracellular domain and their functional effects. We show that one antibody (Y4) with the highest affinity binding, induces channel internalization. The addition of Y4 to KCNK9-expressing carcinoma cells reduces cell viability and increases cell death and systemic administration of Y4 effectively inhibits growth of human lung cancer xenografts and murine breast cancer metastasis in mice. Evidence for Y4-mediated carcinoma cell autonomous and immune-dependent cytotoxicity is presented. Our studies reveal that antibody-based KCNK9 targeting is a promising therapeutic strategy in KCNK9-expressing malignancies. Moreover, our strategies should be generally applicable to targeting KCNK9 in other pathologies and for targeting other ion channels.

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Chapter 1

Introduction

Overview

Twenty five percent of human genome encodes membrane proteins. Among them, six percent are ion channels. In the most basic terms, ion channels are transmembrane proteins that form a water-filled pore to facilitate the passage of ions along their electrochemical gradient across cellular membranes. Gating, or in other words, the opening or closing of ion channels is sensitive to various stimuli, including thermal fluctuation, mechanic deformation and chemical stimulation. Ion channels, depending on the gating modality and ion selectivity, may be divided into different families. Within the same family, multiple members often display considerable similarity in architecture and/or sequence but serve distinct biological functions [6]. In human, there are sixteen ion channel families. They demonstrate enormous diversity at the sequence, structural and functional levels [6, 7]. The vast number and diversity of ion channels allow them to mediate a broad spectrum of biological processes including nerve and muscle excitation, hormone secretion, cell proliferation, as well as learning and memory. Consequently, defects in ion-channel function often have profound physiological effects. To date, mutations in over sixty different ion channel genes have been found to cause human diseases, also known as channelopathies. They are present in almost every area of clinical neurology, from epilepsy, migraine and movement disorder that involve the central nervous system to various diseases in the periphery [8]. Because of their important functional roles, ion channels make up the second largest class of drug targets in the market [6, 9]. However, despite vigorous therapeutic investigation, ion channels are still under-exploited as
therapeutic targets. Forty percent of ion channels have no known ligands or drugs, and of the remaining sixty percent, many lack specific modulators [6, 9].

**Potassium channels**

Potassium (K⁺) channels form the largest portion of the ion channel proteome. Structurally, they can be divided into three distinct classes – voltage-gated K⁺ channels (Kv), inward rectifying K⁺ channels (Kir), and two pore domain K⁺ channels (K2P). Their topological designs are shown in Fig. 1.1. Each subunit of voltage-gated K⁺ channels contains six transmembrane domains (S1-6) and one pore-forming region (Fig. 1.1a). The presence of positive charges in the S4 domain allows these channels to sense voltage changes across the membrane. Another class, inward rectifying K⁺ channels, consists of two transmembrane domains flanking one pore-forming region (Fig. 1.1b). These channels allow K⁺ ions to pass more easily into the cells than out of cells, giving rise to an inward current. Both voltage-gated and inward rectifying K⁺ channels form functional tetramers, where four subunits assemble by homomeric or heteromeric association to form a single ion pathway. Two pore domain K⁺ channels, also known as K2Ps, are the newest members of K⁺ channel superfamily. Unlike canonical K⁺ channels, K2Ps form

![Figure 1.1 Topology of potassium channel subunits](image)

(a) Voltage-gated potassium channels.
(b) Inward-rectifying potassium channels.
(c) Two pore domain potassium channels.
Each subunit consists of four transmembrane domains and two pore-forming regions (Fig. 1.1c). One signature feature of K2P channels is a large extracellular loop between transmembrane domain 1 (M1) and pore-forming region 1 (P1), referred as the M1P1 loop. Functionally, K2P channels mediate background or leak currents that are critical for the maintenance of resting membrane potential in both excitable and non-excitatory cells.

The first human K2P channel was cloned in 1996. Since then, this family has embraced another fourteen members, which can be subdivided into six subfamilies based upon sequence similarity and functional resemblance (Fig. 1.2). One of these subfamilies is TASK (TWIK-related acid sensitive family of K+). There are three TASK channels – TASK1, TASK3 and TASK5, also referred as KCNK3, KCNK9 and KCNK15. As indicated by their names, TASK channels are sensitive to extracellular pH [11, 12]. To date, recombinant KCNK15 has yet to be expressed functionally, hence its function in native cells is unknown. KCNK3 and KCNK9 are the closest related members at the sequence and molecular levels. Their conductance is inhibited upon extracellular acidification, and they respond to some external regulators similarly [11, 12]. In addition, these are the only K2P subtypes that are able to form functional heterodimers, although the significance of this association is unclear. Under physiological conditions, KCNK9 is mainly expressed in tissues of the central nervous system (CNS) (Fig. 1.3). For instance, in cerebellum KCNK9
regulates background currents that fine-tune the action potential firing of cerebellar granule neurons. Overall, KCNK9 has low abundance in peripheral tissues. One exception is the adrenal gland where KCNK9 expression is restricted to the glomerulosa layer and influences aldosterone secretion [13]. In comparison, KCNK3 shows broad expression in the CNS and periphery (Fig. 1.3). Tissue expression pattern of KCNK9 is well conserved from lower mammals to human [14]. Besides genetic regulation, KCNK9 expression is also subject to epigenetic regulation. Maternal imprinting of KCNK9 has been found in human brain and mammary tissues [15, 16]. Loss of imprinting is reported in breast cancer patients, leading to an increase of copy number [17, 18].

**KCNK9 and disease**

Over the years, malfunction or altered expression of KCNK9 has been associated with various disorders. For instance, a missense mutation within KCNK9 leads to a dominant negative mutant that results in mental retardation originally identified in an Israeli Arab family [19]. However, knocking out KCNK9 in

---

**Figure 1.3 KCNK9 and KCNK3 mRNA expression in human tissues**

*Adapted from Medhurst, AD et al. 2001*
rats show little developmental impairment [11, 12]. This could be due to compensatory effects of other K2P channels, or the need of defects in additional genes or concurrent stressors to cause a severe phenotype. Some mild phenotypes were observed in knockout animals including sleep dysregulation and abnormal aldosterone secretion [20, 21].

KCNK9 has gained considerable interest owing to its role in promoting tumorigenesis. The first line of evidence came from genomic studies where high resolution mapping of a novel amplicon in human breast and lung cancers revealed KCNK9 as the only gene encoded in this region. The KCNK9 loci showed 3~10-fold genomic amplification in 10% of breast cancers, along with up to over 100-fold mRNA up-regulation in about 40% of breast and lung cancers. Increase in copy number and transcript is consistent with elevated protein detected in tumor tissues [22, 23]. In follow-up studies, enforced KCNK9 expression conferred tumorigenicity to otherwise nontumorigenic murine mammary gland epithelial cells and embryonic fibroblasts in nude mice, possibly by facilitating cell survival under hypoxic or serum-deprived conditions [22, 23]. Furthermore, this malignant transformation is dependent on the function of KCNK9, as introduction of a dominant negative mutant eliminated the tumor-promoting capacity of wild type KCNK9 [23]. Since these initial studies, KCNK9’s oncogenic properties have been explored in multiple cancer cell line models [22-25]. However, how endogenous KCNK9 contributes to neoplasia and its potential as a therapeutic target remain elusive due to the lack of specific modulators. Moreover, depending on the cancer type and method used to manipulate channel function, different roles of KCNK9 were implicated in cancer. For example, inhibiting KCNK9 via dominant negative mutant, anti-sense shRNA or non-specific chemical blockers suppressed
proliferation of lung carcinoma and melanoma cells. In contrast, pharmacological manipulation of KCNK9 with chemical modulators known to lack subtype specificity led to contradictory results in glioma cell lines [25]. Differences in these in vitro studies could be attributed to differences among cancer types or off-target effects of chemical modulators. This highlights the need for highly selective pharmacological agents to investigate KCNK9 function.

**Conventional pharmacological modulators**

Conventional strategies of chemical screens often cannot focus on a specific region within the target protein, making them inefficient for identification of high specificity modulators. Therefore, pharmacological profiling is particularly difficult for proteins with highly homologous architecture, such as ion channels. K2P channels present more challenges, as their M1P1 loop (shown in orange in Fig. 1.4) forms a structured cap that sits directly on top of the selectivity filter, rendering them insensitive to many classical potassium channel blockers, such as TEA, 4-aminopyridine, and quinidine [11, 12]. High throughput chemical screens have been carried out to identify novel KCNK9 modulators [26-28]. Table 1 lists the most potent compounds that have been reported so far. Despite their high potency,
these compounds demonstrate only moderate selectivity, which limits their utility in functional studies and clinical translation.

Table 1 Comparison of novel KCNK9 chemical modulators

<table>
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<tr>
<th>Compounds</th>
<th>KCNK9 IC$_{50}$ µM</th>
<th>KCNK3 IC$_{50}$ µM</th>
<th>Selectivity KCNK3 IC$<em>{50}$/KCNK9 IC$</em>{50}$</th>
</tr>
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<tr>
<td>C23</td>
<td>0.035</td>
<td>0.017</td>
<td>0.5</td>
</tr>
<tr>
<td>A1899</td>
<td>0.45</td>
<td>0.23</td>
<td>0.5</td>
</tr>
<tr>
<td>SID134418982</td>
<td>0.41</td>
<td>3.16</td>
<td>7.7</td>
</tr>
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Antibody development

Antibodies, produced by B cells, are essential players in humoral immune defense against foreign antigens. The vast repertoire of binding pockets, known as variable regions of antibodies, is generated by recombination of V, D, J segments. Their affinity is improved over time through a process of somatic maturation,

Figure 1.5 Road map of antibody therapeutic development
Adapted from Weiner, LM et. al 2010 [5]
known as affinity maturation. These unique processes are the basis for antibodies’ high specificity and affinity. Antibody recognition epitopes often consist of 5–8 amino acids sufficiently specific to identify any given molecular entity. The development of antibody therapeutics has come a long way since the initial proposal of the magic bullet concept by Paul Ehrlich in the late 19th century. Thanks to technological advancements such as hybridoma technology and phage display platform, a variety of antibody-based therapeutics with improved safety and efficacy have been developed for the treatment of human diseases (Fig. 1.5) [29, 30]. Now, antibodies represent the fastest growing class of therapeutics [31]. They have been broadly used to target cell surface receptors and antigens, especially as applied to cancer treatment [30].

**Antibody-based targeting of ion channels**

Antibodies are commonly used in ion channel research as detection reagents or for structural studies. Utilization of antibodies to manipulate ion channel activity is not well explored. No ion channel targeting antibodies have been approved for clinical use (Fig. 1.6). Is it feasible to target ion channels using antibodies? In order for this strategy to work, epitopes within the channel protein need to be accessible, specific, and functional. Figure 1.7 summarizes the length of extracellular domains (ECD) for different channel families. Overall, ion channels have 30–700 amino acids on the extracellular side. This means if a contiguous epitope consists of 10

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**Figure 1.6 Approved therapeutic antibodies that target membrane proteins [4]**
amino acids, for a given channel, there are 20~690 possible epitopes, representing a sufficiently large repertoire accessible to antibodies. In addition, sequence within the ECD is often not well conserved among members within the same channel family, providing desirable variation for subtype-specific targeting by antibodies. Importantly, the ECD of ion channels also plays essential functional roles, as exemplified by studies of toxins. For instance, psalmotoxin-1 binds to ASIC1 channel ECD, increases H⁺ affinity and stabilizes the channel in a desensitized state [32]. Agitoxin-2 on the other hand, binds to the outer pore of Kv channels and directly blocks ion entry [33]. Hence, antibodies not only can bind to ion channel ECD but exert functional effects.

In human, antibody binding to ion channels and consequent functional preturbation have been found to cause various autoimmune diseases (Table 2). The roles for these autoantibodies in pathogenesis are implicated by correlations between antibody titer and severity of disease, effectiveness of plasma exchange,

![Figure 1.7 Length of ion channel extracellular domains](image)

and/or immunosuppressive therapy. There are several well-documented cases. One of them is paraneoplastic channelopathy where autoimmune neurological...
disorders coexist with tumors, and tumor antigens are indicated as the trigger for autoantibody generation. In these instances, autoantibodies are utilized as diagnostic or prognostic markers for both neurological diseases as well as cancers (see review [34]). For example, in some patients with acquired neuromyotonia, also known as Isaac’s syndrome, self-reactive antibodies against the Kv1 voltage-gated potassium channel prevent membrane re-polarization, increase acetylcholine release, and prolong compound action potentials. As a result, excess release of acetylcholine leads to muscle twitching, cramps, stiffness, and abnormal muscle contraction and relaxation. Experimental evidence suggests that these Kv1 antibodies act by inducing channel internalization, leading to a reduction of functional channels on the cell surface [35]. Autoantibodies targeting ligand-gated ion channels have also been reported. Myasthenia gravis is a prototypic autoimmune channelopathy caused by antibodies against nicotinic acetylcholine receptors at the neuromuscular junction. This autoimmune attack leads to accelerated receptor internalization, complement activation and aberrant ligand binding. These naturally occurring ion channel targeting antibodies serve as a “proof of concept” for manipulating ion channel activity using antibodies.

As early as three decades ago, antibody modulators were generated and noted for their utility in functional studies of voltage-gated ion channels. Monoclonal antibodies (mAbs) were raised against membrane fragments from eel electroplax enriched for voltage-gated sodium channels [36, 37]. Among these antibodies, SC-66-5 and SC-72-14 were found to attenuate the action potential in rat nerve fibers. SC-66-5 inhibited depolarization and prolonged the repolarization phase. SC-72-14, on the other hand, altered the voltage-dependence of channel inactivation and inhibited sodium current [37-39]. In a latter study, SC-72-14 was
also found to reduce Vmax and membrane responsiveness of canine cardiac fibers [40]. Another antibody, SC-72-38, enhanced the excitability of rat muscle cells by changing the voltage-dependence of sodium channel activation and inactivation. The observed changes were similar to those induced by Tityus serrulatus toxin γ (TiTXγ) which was also biochemically competitive with SC-72-38 [41]. Effects of these mAbs include rapid modulation of action potential and/or internalization of sodium channel proteins [38, 39]. In another study, a mAb against dihydropyridine (DHP)-binding complex in rabbit muscle transverse tubules was made and it suppressed the slow calcium current in a mouse muscle cell line, shifting the voltage of activation threshold to a more positive value and slowing down the rate of inactivation [42, 43].

To gain higher preference for subtype selectivity and harness the benefits of human genome sequence data, instead of injecting homogenized membrane fragments, synthetic peptides or recombinant proteins corresponding to a specific region in the channel is used to generate antibodies. Regions in close proximity to the pore are of particular interest, since perturbation through antibody binding is likely to affect conductance. An anti-peptide antibody against a region C-terminal to the pore of voltage-gated calcium channel α1D subunit effectively reduces 40% of native L-type calcium currents in dorsal root ganglion (DRG) neurons and cardiac myocytes under depolarizing conditions. Antibodies can also be generated against the region N-terminal to the pore. This region is easily accessible and in many cases it displays considerable sequence variation. This strategy, now known as E3 targeting (named under third extracellular loop), is used in the studies of several voltage-gated cation channels [44-46] [47]. Antibodies that specifically target the E3 domain of Kv1.2 or Kv3.1 inhibited whole cell currents in neuronal
cells with an IC$_{50}$ close to 60 nM [47]. Antibodies against Nav1.5 and TRPC5 E3 domain inhibited whole cell currents up to 60% in cells transfected with corresponding channels [46]. E3-targeting antibodies of P/Q- and N-type voltage-gated calcium channels effectively attenuated presynaptic calcium currents and excitatory postsynaptic currents. Cerebellum infusion of these antibodies caused cerebellar ataxia phenotypes in mice, establishing a link between voltage-gated calcium channels and pathogenesis [48]. Besides regulating excitability, some E3-targeting antibodies could modulate store-operated Ca$^{2+}$ entry [49], oligodendrocyte progenitor cell proliferation and migration [50], and tumor growth [51].

Besides regions in close proximity to the ion pore, other functional modalities have also been explored as target regions of antibodies. Polyclonal antibody to the voltage sensor of sodium channel (anti-C$^+$) shifted the voltage dependence of fast-FA currents to a more negative value and increased the inactivation rate of slow currents in cultured DRG cells [52, 53]. Moreover, its binding to rat brain synaptosomal vesicles was enhanced by depolarization, suggesting a depolarization-induced conformational change that made the epitope more accessible to the antibody [54]. NESOpAb, a Nav1.5 antibody that targets the second extracellular loop, inhibited sodium currents with an IC$_{50}$ of less than 25 nM. It also exhibited exquisite selectivity, distinguishing neonatal and adult splice variants which differ by seven amino acids only [55]. Table 3 is a summary of ion-channel targeting antibodies.

**Targeting KCNK9 using antibody modulators**

There are three structurally distinct classes of K$^+$ channels. Fig. 1.8 shows the crystal structure of three mammalian K$^+$ channels, each representing one of the three classes. If we compare them side-by-side, a major feature that distinguishes
K2P channels from others is the large M1P1 loop (highlighted in blue box). Besides extracellular accessibility, sequence within this loop is not well conserved among different subtypes. The subtypes ranges from 6% to 46% (Fig. 1.9). Functionally, the M1P1 loop serves as a physical guard of ion permeation pathway. Additionally, several sites within or in vicinity of the M1P1 loop are important for regulating channel gating. Site-directed mutagenesis reveals that glutamate 30 (E30), glutamate 70 (E70) and histidine (H98) control KCNK9 open probability and sensitivity to external stimuli [11, 12]. Therefore, antibody binding to the M1P1 loop could allow us to achieve subtype-specific manipulation of KCNK9 channel function.

**Summary**

Ion channels are important regulators of cellular functions. KCNK9 is a member of K2P channels that mediate background currents in both excitable and
non-excitable cells. Over-expression of KCNK9 has been observed in various human solid tumors and its expression level inversely correlates with survival of patients with squamous cell lung and breast cancers. To investigate the functional significance of KCNK9 and its potential as a therapeutic target, strategies that afford subtype-specific manipulation are essential. So far, no selective chemical modulators has been identified for KCNK9. In this study, we propose to develop antibody modulators targeting the M1P1 loop of KCNK9 and use them as tools to explore the biologic function and therapeutic potential of KCNK9. Figure 1.10 is a roadmap that summarizes the process of antibody development and characterization.
Figure 1.10 Roadmap of mAb development and characterization
Chapter 2

Development of KCNK9-specific Monoclonal Antibodies

Overview

One challenge in developing antibodies against ion channels is the generation of antigens because it is difficult to express and purify large-scale transmembrane proteins with native structure preservation. People used to immunize animals with membrane fragments or native protein extracts. These antigens represent a mixture and often lead to antibodies that are highly heterogeneous and lack target specificity. Alternatively, antibodies can be raised to peptides synthesized in vitro. Many features can be added to peptides to optimize efficient production and purification. However, peptides are very small and do not undergo in vivo processing as proteins. Hence, antibodies generated in this way have limited diversity, as they are restricted to species that recognize short linear epitopes whereas antibodies that bind conformational epitopes or long linear epitopes would be missed. Another well-established method is to make recombinant proteins where a large segment of protein domain is fused to a vector, which allows the fusion protein to be expressed either via fermentation or in mammalian cells. In this way, antigens are more likely to resemble the native form. With high purity peptides or proteins, in vivo immunization is commonly used to generate antisera to make advantages of hypermutations and affinity maturation [56]. Either polyclonal (pAb) or monoclonal (mAb) antibodies can be obtained. The invention of hybridoma technology in the 1970s revolutionized the production of mAbs. Hybridomas derived from fusion of spleen cells from immunized individuals with immortalized myeloma cells provide a continuing source of antibodies of single specificity [57]. Subsequently, hybridomas can be screened and subcloned to
obtain single clones. Screening is largely based on antigen binding as determined by biochemical assays including enzyme-linked immunosorbent assay (ELISA), western blot and flow cytometry. A functional screen is usually performed after mAbs have been purified from selected single clones. This is because the active concentration of mAbs in hybridoma supernatant is sufficient to detect binding but often insufficient for functional investigation, especially in assays used to study ion channel physiology. In addition, ion channel assays have stringent buffer requirements and nonpurified supernatant could contain components that significantly interfere with assay readout.

In this chapter, I will discuss the development of KCNK9 mAbs through antigen generation, hybridoma production, screening, and biochemical characterization.

**Results**

**Expression of recombinant antigens**

To generate antigens that recapitulate native human KCNK9 (hKCNK9) structure, the M1P1 loop was expressed as a recombinant protein in mammalian cells to optimize preservation of 3D structure and post-translational modifications. We used two expression strategies developed by Dr. Lieping Chen at Yale University and Dr. Daniel Leahy at the Johns Hopkins University [58, 59]. We fused hKCNK9 M1P1 loop either to murine IgG2A constant regions, designated as K9M1P1-mIgG (Fig. 2.1a); or to human growth hormone, designated as hGH-K9M1P1 (Fig. 2.1b). Both vectors allow expression in mammalian cells and secretion into culture medium for collection and purification. We transiently transfected HEK293T cells with hKCNK9-mIgG using lipofectamine, or CHO-S cells with hGH-hKCNK9 using polyethylenimine (PEI). We validated fusion protein expression by western blotting
K9M1P1-mlgG transfection is relatively straightforward – adherent HEK293T cells were transfected with lipofectamine:DNA (ratio 3:1) for 24 hours following manufacturer’s recommendation. To express hGH-K9M1P1, CHO-S cells were first adapted from adherent to suspension culture. Extensive optimization involving transfection medium, time, ratio of PEI versus DNA, and seeding cell density was carried out to improve expression efficiency. Eventually, PEI:DNA (ratio 2:1) was used to transfect CHO-S cells at 2 µg/million cells in hybridoma serum free medium. We collected conditioned medium 96 hours post-transfection for subsequent purification.

**Figure 2.1 Antigens generated for mAb development**
(a) K9M1P1-mlgG design. Antigen (~37kDa) and vector (~25kDa) expression verified by western blotting.
(b) hGH-K9M1P1 design. Antigen (~37kDa) and vector (~25kDa) expression verified by western blotting.

**Purification of recombinant antigens**
K9M1P1-mlgG was affinity purified using protein A agarose beads. Optimization involves loading volume, bead volume, incubation and wash time, pH of wash and elution buffers. Optimal protocol is: equilibrate beads with sodium acetate buffer pH5.0. After loading conditioned medium (100ml medium/ml beads) from HEK293T cells, we collected flow through to examine binding efficiency, and
then we washed beads with sodium acetate buffer pH5.0 three times to remove non-specific binders. Each wash flow through was collected to examine wash stringency. Bound antigens were eluted with glycine hydrochloride buffer pH3.0 and neutralized with Tris buffer pH8.0. Purified antigens were dialyzed against PBS. Analysis of loading medium, flow through and elution by western blotting, average yield of K9M1P1-mlgG purification was ~80%. Purity reached ~85% based on silver staining and densitometry analysis (Fig.2.2a). The other antigen, hGH-K9M1P1 contains a His-tag that allows affinity purification using nickel beads. Optimization involved loading volume, bead volume, wash time, imidazole concentration of wash and elution buffers. Beads were equilibrated with PBS and 15 mM imidazole. After loading, beads were washed with 15 mM imidazole once and eluted with 300 mM imidazole followed by dialysis against PBS. Similar to K9M1P1-mlgG purification, loading medium, flow through, wash and elution were collected to analyze the efficiency of purification. Average yield of hGH-K9M1P1 was ~80% and purity was ~87% (Fig.2.2b).

![Affinity purified recombinant antigens](image)

**Figure 2.2 Affinity purified recombinant antigens**
(a) K9M1P1-mlgG silver staining.
(b) hGH-K9M1P1 silver staining.
Hybridoma generation and screening

The M1P1 loop of human and mouse KCNK9 differ by five amino acids only. Hence, K9M1P1-mlG is poorly immunogenic in normal murine strains. In order to improve the immunogenicity, we immunized NZB/W x NZB/W mice. This is a murine systemic lupus erythematosus model and its hypersensitive immunity serves as an advantage to generate antibodies against murine self-antigens or highly conserved human antigens [60]. hGH-K9M1P1, on the other hand, was injected into BalB/C mice. For both strains, we immunized with 100 µg protein and Freund’s adjuvant subcutaneously. After two rounds of immunization, we bled mice to test serum titer by ELISA and flow cytometry using HEK293T cells transiently transfected with K9M1P1-TM-eGFP. After serum titer reached 1:10,000 by ELISA and 1:100 by flow analysis, we performed a final antigen boosting and sacrificed mice to collect spleen cells. We fused spleen cells with myeloma cells to generate fusion clones. In total, we obtained ~2,500 fusion clones. 10 days after fusion, we screened supernatants by ELISA and 60%~80% of the fusion clones were positive. We then validated these positive clones further by flow cytometry analysis to evaluate capacity to bind to cell surface KCNK9 and ~15% were positive in this assay. We performed multiple rounds of limiting dilution cloning to obtain single clones of high reactivity based on ELISA and flow cytometry analysis. In the end, we obtained 40 single clones – 4 raised to K9M1P1-mlG, designated as Y-mAbs and 36 raised to hGH-K9M1P1, designated as H-mAbs, and scaled them up for mAb generation and purification. Antibodies were affinity purified using protein G agarose beads following manufacturer’s recommendations.

Characterization of mAb specificity and affinity
All mAbs were IgG1 and demonstrated a subtype-specific binding to hKCNK9 over other K2P subtypes including hKCNK3 – the closest related member of hKCNK9 (Fig. 2.3a, b, c). Y-mAbs displayed nanomolar to sub-nanomolar affinity.

Figure 2.3 Y4 binds to hKCNK9 with subtype specificity and high affinity
(a) Western blot analysis of hGH-hK2PM1P1 blotted with Y4 and anti-hGH antibodies.
(b) Flow cytometry analysis of HEK293 cells transiently expressing full-length hKCNK9 or hKCNK3.
(c) Western blot analysis of Y-mAbs against hGH-hKCNK3 and hKCNK9-alkaline phosphatase. Anti-hGH antibody was control.
(d) Sequence alignment corresponding to the M1P1 loop of hKCNK9 and mKCNK9 with nonconserved residues highlighted (top). Binding kinetics of Y-mAbs determined by Octet platform (bottom).
(e) Y-mAbs' differential affinity to human versus murine KCNK9 validated by western blot and (f) flow cytometry analysis of HEK293 cells transiently expressing hKCNK9 or mKCNK9.
to recombinant hKCNK9 protein with Y4 having the highest affinity (K_D~0.7 nM). Y-mAbs also showed cross-reactivity with recombinant murine KCNK9 (mKCNK9) with a lower affinity (Fig. 2.3d). The degree of cross-reactivity was different among the Y-mAbs (Fig. 2.3e, f). This suggests Y-mAbs recognize distinct epitopes and their locations can be inferred based on sequence alignment.

**Discussion**

Within this chapter, I have described the development of mAbs recognizing the M1P1 loop of KCNK9. We generated recombinant antigens in two formats. Fusion to murine IgG2A ensures that antibodies are specifically raised to the hK9M1P1 region. In addition, mIgG2A can stimulate T cells to enhance humoral responses against fusion proteins [61]. However, because sequence within the M1P1 loop of human and mouse KCNK9 is highly conserved, hK9M1P1-mIgG has poor immunogenicity in mice. As a result, we used a mouse strain with defective immune tolerance to improve immunogenicity to obtain desirable serum titer. Alternatively, fusion to human growth hormone makes hGH-K9M1P1 sufficiently immunogenic in mice. One disadvantage is that significant antibodies are generated to the hGH region and stringent counter screen is needed to select for antibodies that recognize the K9M1P1 region. Hence, depending on the antigen format, optimization of immunization and subsequent screens is critical.

After we made fusion clones, we used two-tier screening to identify hybridomas that produce high affinity antibodies. The first screen used serially diluted supernatants from hybridomas by ELISA. Hybridomas from hK9M1P1-mIgG immunized animals were tested against purified mIgG, hGH-K3M1P1, hGH-K9M1P1, and K9M1P1-AP; whereas, hybridomas from hGH-K9M1P1 immunized animals were tested against purified hGH, K3M1P1-mIgG, K9M1P1-mIgG, and
K9M1P1-AP. We ranked ELISA reactivity at 1:10,000 dilution and picked the top 100 clones with specific binding to K9M1P1. To select for antibodies that bind KCNK9 on the cell surface, we used flow cytometry analysis as the secondary screen. Supernatant from early stage fusion clones and subclones is highly heterogeneous and the active concentration of antibodies is low, thus, robust channel expression is required to achieve desirable sensitivity of flow-based screen. We tested different KCNK9 stable and transient transfectants with commercial KCNK9 antibodies. However, none of the cell lines showed strong staining as analyzed by flow cytometry. Hence, we made a construct fusing the K9M1P1 loop to an artificial transmembrane domain and eGFP. This fusion protein can be efficiently expressed and trafficked onto the cell surface upon transient transfection. eGFP signal allows us to gate specifically on transfected cells during flow cytometry analysis. We used K9M1P1-TM-eGFP expressing cells to screen clones from initial fusion and during the first two rounds of subcloning. Each time we selected 10 clones based on reactivity to proceed to the next step. Clones with nonspecific binding to parental HEK293T cells or eGFP-negative transfected cells were removed. For the final round of subclone screening, we included a tetracycline (Tet)-inducible stable cell line as a more stringent selection criterion. Clones with desirable reactivity to Tet-induced cells and minimal binding to noninduced cells were scaled up to make antibodies. In summary, specificity and sensitivity are important factors to consider designing screening assays. To ensure specificity proper controls help eliminate false positives and low affinity antibodies. Giving the heterogeneity of hybridoma mixture, it is helpful to maximize assay sensitivity and gradually increase the stringency of selection criteria to avoid losing false negative clones.
**Experimental procedures**

**Cell culture**

HEK293 and HEK293T cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-Glutamine (L-Gln). HEK293-KCNK9 stable cell lines were previously generated and KCNK9 expression was induced by incubation with 1 µM tetracycline for 16 hours. These cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-Glutamine, 400 µg/ml hygromycin, and 15 µg/ml blasticidin. CHO-S freestyle cells were maintained in hybridoma serum free medium supplemented with 10% FBS and 2 mM L-Gln. All cells were maintained at 37 °C in 5% CO2.

**Western blot**

Western blot was performed using quantitative Western blot system (LI-COR Bioscience, Lincoln, NE, USA) following the manufacturer’s instructions. Membranes were blotted with hGh (Abcam), KCNK9 (Alomone), and KCNK9 antibodies made in house. Secondary antibodies were labeled with IRDye infrared dyes (LI-COR Biosciences, Lincoln, NE, USA) and protein levels were quantified using the Odyssey Infrared Imager (LI-COR Biosciences).

**Flow cytometry**

Single-cell suspensions were labeled with KCNK9 mAbs for 30 minutes at 4 °C. After washing with ice-cold PBS, cells were incubated with APC-conjugated goat anti-mIgG secondary antibody for 30 minutes at 4 °C. Cell staining was analyzed using FACS Calibur flow cytometer (BD Biosciences) or sorted using FACS Aria-II flow cytometer (BD Biosciences).

**Antibody binding affinity measurement**
Affinity of binding to recombinant human and murine K9M1P1 was determined using Octet® system (Pall Life Sciences). Association and dissociation rates were measured in real-time by incubating probes coated with Y-mAbs in the presence of hGH-hK9M1P1 or hGH-mK9M1P1. $K_D$ was estimated by deriving association (Kon) and dissociation (Koff) constants from real-time measurement and calculated by: $K_D = \text{Kon/Koff}$. 
Chapter 3

Novel mAbs Inhibit KCNK9 Channel Activity

Overview

Another challenge in generating ion channel-targeting antibodies is the lack of well-established assays for functional screen and characterization. In consequence, hybridomas are screened first based on antibody binding to the target, functional validation is performed after mAbs have been made and purified. Electrophysiological endpoints are the gold standard method to examine chemical ion channel modulators. However, a few limitations to studying antibody effects on KCNK9 electrophysiology were anticipated. First, KCNK9 is constitutively open, generating a K⁺ current similar to cellular leakage. Hence, patch clamp recording using a perfusion protocol only allows 15~20 minutes’ measurement of currents. This may not give sufficient time for antibodies to alter channel activity since Abs have a slower diffusion rate than chemical compounds and they may act by a mechanism that requires long incubation. An alternative approach is a pre-incubation protocol in which the effects of treating two cell populations with control mAb and KCNK9 mAb are compared. However, KCNK9 current has significant cell-to-cell variation. Unless antibody modulators are highly potent, quantitative analysis of two populations is difficult. Fluorescence-based ion influx assays are commonly used for chemical screens due to their high throughput and amenability. Fluorescence Ca²⁺ influx
assay was used to characterize inhibitory antibodies targeted to TRPC5 channel [46]. Our lab developed a fluorescent ion influx assay for high throughput KCNK9 chemical screen. It utilizes the endogenous permeability of KCNK9 channel to thallium (Tl⁺) [26]. Cells were first loaded with a Tl⁺-sensitive dye. Upon stimulations, channels open and Tl⁺ ions flow into cells along their electrochemical gradient. When Tl⁺ is chelated by the dye, a fluorescence change at 525nm can be observed and monitored over time. Recorded signal is proportional to the number of open channels on the cell surface. This assay was first developed by David Weaver for K⁺ channel chemical screens [62]. Superior sensitivity, specificity and throughput of this assay make it desirable for screening. In addition, this assay affords a lot of flexibility in terms of incubation time, assay buffer, temperature, which allowed us to optimize assay conditions for identification and characterization of antibody modulators.

In this chapter, I will discuss the utilization of Tl⁺ influx assay for functional screen and mechanistic studies of KCNK9 antibody modulators.

**Results**

**Assessment of mAb effects in thallium influx assay**

A Tet-inducible KCNK9 stable cell line was previously generated for a chemical screen using the Tl⁺ ion influx assay. All 40 mAbs were capable of recognizing KCNK9 expressed on the cell surface of this cell line (Fig. 3.2). We dialyzed

![Figure 3.2 Flow cytometry analysis of cell surface hKCNK9 in tetracycline (Tet) inducible stable cell line stained with Y1 and Y4 in the presence (+K9) or absence (-K9) of induction](image)

Parental HEK293 cells were used as negative control.
purified antibodies against Tl⁺ assay buffer and tested these mAbs in Tl⁺ influx assay using a pre-incubation protocol as outlined in Fig.3.3a. Upon tetracycline induction, cells were treated with 0.4 mg/ml mAbs for 36 hours. 6 of 40 mAbs were effective in inhibiting KCNK9 conductance (Fig. 3.3b). This inhibition was not due to differences in cell viability as indicated by comparable fluorescence intensities at time 0 of fluorescence measurement (Fig. 3.3c). Y4 was most potent showing up to 30% inhibition of Tl⁺ signal. Hence, we chose Y4 for follow-up studies. Time-course experiments indicated that Y4-mediated channel inhibition was significant after 6-hour incubation. The inhibitory effect was stronger after longer exposures (Fig. 3.4a) and it was dependent on Y4 concentration (Fig. 3.4b). We also set up whole cell patch clamp recordings to examine the possibility to acutely block of KCNK9 by Y4, since KCNK9 is sensitive to extracellular pH and the corresponding modulatory residue locates in close vicinity of the M1P1 loop. Thus, we also used electrophysiological assays to examine Y4’s effects on pH sensitivity. We perfused cells with 0.4 mg/ml Y4 at pH7.4 and pH6.5, and recorded the currents for 20 minutes. Y4 had no detectable effects on conductance at pH7.4. Acidification suppressed KCNK9 conductance by 60%, and Y4 treatment did not alter this pH effect (Fig. 3.4d).
**Figure 3.3 MAb-mediated inhibition of Tl⁺ conductance**

(a) Outline of Tl⁺ assay adapted for mAb screening.
(b) Representative Tl⁺ traces of 36-hour treatment with Y2, H8, H10, H14 and H30 mAbs that showed marginal inhibition.
(c) Fluorescence at time 0 did not show significant differences upon mAb incubation at indicated time periods suggesting no dramatic differences in cell/channel number.
Figure 3.4 Y4 inhibits KCNK9 conductance in a time-dependent manner
(a) Representative Tl\(^+\) traces plotted as fluorescence change over time. \(n=24\), *\(P<0.0001\), two-tailed Student t-test. Y4 – blue line, PBS-black line.
(b) Time-dependent inhibition of Tl\(^+\) conductance by Y4. Tet\(^+\) cells were treated with Y4, Y1 or PBS prior to Tl\(^+\) assay and compared to cells without treatment. Difference in Tl\(^+\) conductance at time point 80 was used for comparison. \(n=24\), *\(P<0.0001\), ANOVA.
(c) Concentration-dependent inhibition of Tl\(^+\) conductance by Y4. \(n=6\), *\(P<0.01\), two-tailed Student t-test.
(d) Electrophysiological patch clamp recording protocol used to examine Y4’s acute effects on hKCNK9 and quantification of conductance in the presence of Y4. KCNK9 current was normalized to recording at pH7.4.
Characterization of mAb-induced internalization

It is known that when antibodies interact with cell surface antigens, they often induce internalization and endocytosis of antigens from the cell surface [63-65]. Given the time dependence of Y4's inhibitory effects, we speculated that antibody-induced endocytosis is one mechanism of actions of Y4 action. We first examined this possibility using flow cytometry analysis. Upon tetracycline induction, we treated KCNK9-expressing HEK293 cells with Y-mAbs for 36 hours at 30 °C, and channels remaining on the cell surface were stained using a polyclonal KCNK9 antibody examined, as outlined in Fig. 3.5a. Flow cytometry analysis showed that 15.6% KCNK9-positive cells completely lost cell surface signal and became KCNK9-negative; and among cells that remained KCNK9-positive, their overall fluorescence decreased by 25.7%. Reduction of cell surface KCNK9 occurred only in response to Y4 treatment but not to treatment with other Y-mAbs (Fig. 3.5a, b). Similar to the effect of Y4 on inducing the internalization of transgenic hKCNK9 in HEK293 cells, we found that Y4 induced internalization of endogenous KCNK9 in a lung cancer cell line with 21.7% reduction of KCNK9-positive cells and 9.8% reduction of overall fluorescence in KCNK9-positive cells (Fig. 3.5e-g). For further analysis, we used confocal microscopy to track fluorescently labeled Y4. Fluorescent conjugation of Y4 did not alter its binding affinity to the cell surface channel (Fig. 3.6a). We tested a variety of cell lines and transfection strategies, and found that transiently transfected COS-7 cells provide most desirable morphology and viability for imaging. Since endocytosis is temperature dependent, we treated cells with Y4 at 4°C for 3 hours as a control and induced endocytosis by 12-hour incubation at 30°C. We found that Y4 treatment led to a reduction of surface signal along with increased intracellular signal, predominantly visible in the perinuclear
region. This was observed only at a temperature permissive to endocytosis. For quantitative analysis, we studied the co-localization of Y4 with EEA1, which is an
early endosome marker often used in endocytosis studies. Image analysis showed significant (r=0.73±0.15) co-localization of Y4-stained KCNK9 and RFP-tagged EEA1. This phenomenon was specific to KCNK9 since no change was detected in cells expressing KCNK3 (Fig. 3.6b). Taken together, these data indicate that mAb-induced endocytosis of functional channels from cell surface is one of the mechanisms mediating reduced KCNK9 channel conductance observed in the ion-influx assay.

**Discussion**

A fluorescence-based Tl⁺ assay was adapted to identify antibodies that can modulate KCNK9 activity. The majority of antibodies had no detectable effects in this assay despite their ability to bind cell surface KCNK9. We found Y4, the mAb with highest affinity binding, was most efficacious in inhibiting channel conductance. This further validated the affinity-based screening strategy discussed in Chapter 2.

The time dependence of Y4’s activity suggested to us that antibody-induced endocytosis is a mechanism by which Y4 reduces functional channels on the cell surface leading to a smaller fluorescence signal in the Tl⁺ assay. Follow-up studies
using flow cytometry and confocal imaging supported our hypothesis. There are multiple mechanisms by which antibodies perturb channel function. Most antibodies generated by rational design either occlude the ion permeation pathway or alter modalities essential for channel gating; and their effects that typically become apparent within minutes of antibody addition [6, 7]. Ab-induced

![Graph](image1)

**Figure 3.6 Y4 induces channel internalization from cell surface**

(a) Conjugation of Y4 with Alexa488 did not alter the binding affinity of KCNK9. Tet+ KCNK9 stable cell line was stained with either unlabeled Y4 or Alexa488-labeled Y4 at indicated dilutions followed by a secondary antibody and flow cytometry analysis.

(b) Internalization of KCNK9 from cell surface induced by Y4. COS-7 cells transiently expressing KCNK9 and mcherry or RFP-tagged EEA1 were incubated with Alexa488-conjugated Y4. Cells transiently expressing hKCNK3 and mcherry were control. Nuclei were counterstained with DAPI. Co-localization (arrows) between Alexa488-conjugated Y4 and RFP-tagged EEA1 was analyzed using Imaris software and representative scatter plots were shown. Statistical analysis was based on Pearson’s correlation coefficient calculated from 20 fields. *r>0.5. Bar: 20 µm.
internalization has been reported for several self-reactive antibodies targeting voltage-gated ion channels in paraneoplastic channelopathies such as acquired neuromyotonia and Lambert Eaton syndrome [34]. Tumor antigens are thought to trigger generation of self-reactive antibodies to tumor cell surface proteins including ion channels. Binding of these self-reactive antibodies alone is insufficient to significantly suppress channel function. Instead, long time incubation (2~24 hours) of divalent antibodies is found to induce channel internalization which in turn causes neuromuscular transmission defects [66-69]. In this study, we found that a minimum of 6-hour incubation was required for Y4 to significantly inhibit of KCNK9 conductance. Short time perfusion of Y4 did not show detectable effects as evaluated by electrophysiology assay. This supports down-regulation of cell surface channel expression as one mechanism of Y4’s action, although it does not rule out potential effects on ion permeation and/or gating. An advantage of antibodies that are capable of inducing internalization compared to antibodies that act merely as blockers is that efficacious inhibition depends on the quantity and density of antigens expressed on the cell surface. This offers a mechanism-based specificity such that only tumor cells with sufficiently high channel expression are targeted by antibodies. It is possible that targeting a subset of tumor cells can limit treatment efficacy. However, the fact that Y4 is able to suppress BEN and LX22 tumor growth, as well as 410.4 metastasis despite target heterogeneity supports a balance between specificity and efficacy, and strengthens the potential clinical utility of KCNK9 antibodies. Additionally, optimal patient selection can further improve the effectiveness of KCNK9 targeted therapies. Furthermore, Y4 could have anti-tumor effects independent of KCNK9 function. For example, it can be developed into antibody-drug conjugates where it
facilitates tumor-specific delivery of cytotoxic payloads to kill tumor cells. Also, Y4 may be able to activate effector responses, utilizing the immune system to target tumor cells. In our animal models, Y4 treatment did not achieve tumor regression but rather a reduced growth rate. The effectiveness is comparable to that observed in pre-clinical testing of other therapeutic antibodies [70, 71]. Nevertheless, the mechanism and effector functions of Y4 make it an advantageous treatment strategy with desirable therapeutic index by enhanced specificity and/or efficacy in combination of cytotoxic conjugates or immunotherapies.

**Experimental procedures**

**Thallium (Tl⁺)-based fluorescence assay**

A fluorescence assay previously developed for chemical screen of KCNK9 modulators was adapted for mAbs [26]. In this assay, Tl⁺ served as a surrogate ion for K⁺. Upon tetracycline induction of KCNK9 expression, cells were incubated with 0.4 mg/ml mAbs for 0.5 to 72 hours at 37 °C. After wash-off, cells were loaded with a Tl⁺-sensitive fluorescent dye, FluxOR™ (Invitrogen). Upon channel activation by 2.8 mM K⁺, Tl⁺ influx yielded a fluorescence change monitored as function of time [26]. Parental HEK293 and non-induced KCNK9 cells were negative controls.

**Flow cytometry analysis of channel internalization**

Tet-induced HEK293-KCNK9 cells were incubated with 0.4 mg/ml Y-mAbs for 36 hours at 30 °C to induce endocytosis. KCNK9 channels remained on the plasma membrane were stained with a polyclonal anti-KCNK9 antibody followed by secondary antibody staining and flow-cytometry analysis as described above.

**Confocal microscopic analysis of channel internalization**

Y4 was labeled with Alexa Fluor 488 dye (Life Technologies). COS-7 cells were transiently transfected with hKCNK9 or hKCNK3, and mcherry was co-
transfected as a transfection control. After 36 hours, cells were treated with 0.4 mg/ml Y4 either for 3 hours at 4 °C or for 12 hours at 30 °C. At the end of incubation, cells were washed with PBS, fixed with 4% paraformaldehyde, washed with PBS and mounted with ProLong® Gold Antifade Mountant containing DAPI (Life Technologies). Images were taken at 63X/1.4 with scan zoom 1.0. For colocalization analysis, COS-7 cells were co-transfected with RFP-tagged EEA1 (Addgene). Images were taken at 40X/1.3 with scan zoom 1.0 and quantified using Imaris software (Bitplane). A minimum threshold of red and green channels was selected. The data represent images taken from 20 different areas (n=20).
Chapter 4

Novel mAbs Inhibit Cancer Cell Survival

Overview

KCNK9 over-expression has been reported in a variety of solid tumors, including breast, lung and colon cancers [22, 72]. Partially transformed cells with enforced KCNK9 expression have increased tumorigenicity, forming aggressive tumors in nude mice [22, 23]. Besides over-expression, loss-of-function studies have been carried out to examine endogenous KCNK9 function in cancer cell lines. Inhibition of KCNK9 either via shRNA knock down or a dominant negative mutant was found to inhibit cell viability by 20%–65% [23, 24]. However, these strategies have limited prospects for clinical translation. Given that Y4 is able to suppress KCNK9 function, we expect Y4 to have a similar if not better effect on KCNK9-expressing cells.

In this chapter, I will describe the characterization of endogenous KCNK9 expression in cancer cell lines and patient-derived primary cells, and validation of KCNK9’s oncogenic functions using antibody modulators.

Results

KCNK9 expression and survival analysis

KCNK9 over-expression has been reported in breast, lung and colon cancers [22, 72]. Enforced KCNK9 expression promotes tumor-propagating capacity of non-neoplastic cells and this property is eliminated by co-expressing a dominant negative KCNK9 mutant, suggesting a role for KCNK9 during tumor growth [23]. To assess the clinical relevance of KCNK9, correlations between KCNK9 expression and patient survival were analyzed with commonly used microarray datasets [73, 74]. Patients’ gene expression and clinical data were manually matched. Patients
with available KCNK9 expression data and survival time were segregated into two groups according to KCNK9 expression level – KCNK9 high (two-fold higher than mean) and KCNK9 low (two-fold lower than mean). We found an inverse correlation between KCNK9 expression and overall survival of patients with either squamous cell lung or breast cancer (Fig. 4.1). In squamous cell lung cancer, the 2-year relative survival rates of low KCNK9 expressers increased by 58% compared to high expressers. The hazard ratio between KCNK9 high and low groups is 2.8 (95% CI: 1.222-6.329), meaning the rate of death of KCNK9 high is 2.8 times the rate in KCNK9 low. In breast cancer, the 10-year relative survival rates of low KCNK9 expressers increased by 10% compared to high expressers. The hazard ratio between KCNK9 high and low groups is 1.6 (95% CI: 1.005-2.556). These are very significant and clinically meaningful differences. In both cancer types, the improvement of survival is comparable to other reported prognostic and predictive markers such as p53 [75]. In addition, to strengthen the survival analysis, we performed the same analysis with KCNK3, the closest related

**Figure 4.1 Kaplan-Meier curves for patient survival in relation to gene expression**

n=53 (lung), n=289 (breast), *P<0.05, Log-rank (Mantel-Cox) test.
member of KCNK9, and showed that the survival benefit was KCNK9-specific (Fig. 4.1). This supports the notion that KCNK9 promotes tumor growth and could be a therapeutic target in KCNK9-expressing malignancies.

**KCNK9 expression in cancer cells**

Given that Y4 binds and internalizes KCNK9 in a K2P subtype-specific manner, it affords an advantageous strategy to investigate the role of endogenous KCNK9 in cancer biology. The BEN squamous cell lung cancer cell line was previously shown to have abundant KCNK9 mRNA [23]. We confirmed this at the protein level by western blot and validated cell surface channel expression by flow cytometry (Fig. 4.2). Subsequently, we screened a panel of human (Fig. 4.2) and murine (Fig. 4.3) cancer cell lines as well as patient-derived primary cell cultures by qRT-PCR followed by validation via western blotting or flow cytometry. In addition to BEN, the BT-549 human breast cancer cell line and cells from LX22 patient-derived xenograft (PDX) of small cell lung cancer were found to express KCNK9 at relatively high levels. The MDA-MB-231 human breast cancer cell line, on the other hand, had undetectable expression (Fig. 4.2). mKCNK9 was present in metastatic and non-metastatic murine breast cancer cell lines (Fig. 4.2). Higher expression levels were found in two metastatic cell lines 66.1 and 410.4, suggesting a potential role of KCNK9 in metastasis. Y4 stained 410.4 cells indicating sufficient cross-reactivity with mKCNK9 (Fig. 4b). Interestingly, BEN, BT-549, LX22 and 410.4 cell lines showed two cell populations based on surface staining of KCNK9, designated as K9+ and K9-. K9- BEN cells were stable after in vitro culture; hence, we used them as negative control for subsequent *in vitro* studies (Fig.4.4a). To examine if these two sub-populations of BEN cells possess any intrinsic differences besides KCNK9 expression, we performed cell
Figure 4.2 Endogenous KCNK9 expression in human cancer cells
Figure 4.3 Endogenous KCNK9 expression in murine breast cancer cell lines

(a) BEN and BT-549 cells were FACS-sorted based on hKCNK9 expression and designated as P1 (K9-positive) and P2 (K9-negative) cells. After being passaged three times in vitro, they were stained and analyzed by flow cytometry.

(b) Cell cycle analysis of K9+ and K9- BEN cells based on DNA content. G0/G1: 2N, S: 2N-4N, G2/M: 4N.

Figure 4.4 K9- BEN cells were stable after passaging in vitro
(a) BEN and BT-549 cells were FACS-sorted based on hKCNK9 expression and designated as P1 (K9-positive) and P2 (K9-negative) cells. After being passaged three times in vitro, they were stained and analyzed by flow cytometry.
(b) Cell cycle analysis of K9+ and K9- BEN cells based on DNA content. G0/G1: 2N, S: 2N-4N, G2/M: 4N.
cycle analysis, and K9⁺ BEN sub-population showed 34% enrichment of cells in S+G₂/M phases compared to K9⁻ BEN sub-population (Fig. 4.4b). This suggests that KCNK9 expression and/or function is related to cell cycle progression.

**Inhibition of cell viability**

Inhibition of KCNK9 via a dominant negative mutant or shRNA was shown to inhibit cancer cell proliferation by 20~65% [22-24]. To evaluate if Y4-mediated modulation of KCNK9 has any biological consequences, BEN, BT-549, MDA-MB-231, LX22 and 410.4 cells were treated with antibodies 24-72 hours. Enforced KCNK9 expression was previously shown to improve cell viability under low serum conditions, suggesting a protective effect of KCNK9. Based on this, we speculated that interference of KCNK9 function could have a more pronounced effect under serum deprivation. Hence, we performed cell viability assay at 10% and 0.1% heat-

![Graph showing cell viability of KCNK9-expressing cancer cells](image)

**Figure 4.5 Y4 reduces cell viability of KCNK9-expressing cancer cells**

BEN cells were sorted according to KCNK9 expression level (K9⁺ or K9⁻). n=6, *P<0.01 at 0.1% serum; **P<0.01 at 0.1% and 10% serum.
inactivated serum. Y4 applied at 0.4 mg/ml significantly suppressed the viability of K9+ BEN, BT-549, LX22 and 410.4 cells but not K9- BEN cells or K9- MDA-MB-231 cells (Fig. 4.5). Inhibition of K9+ BEN and LX22 cells was more prominent at 0.1% serum consistent with previous reports.

**Induction of cell death**

Changes in cell viability as determined by MTT assay could result from alterations in cell proliferation, cell death and/or metabolism. To further dissect Y4’s biological effects, we measured L-lactate dehydrogenase (LDH) release as readout of cell death following mAb treatment. Y4 induced significant cell death of

![Graph showing cell death](image)

**Figure 4.6 Y4 increases cell death of KCNK9-expressing cancer cells**

BEN cells were sorted according to KCNK9 expression level (K9+ or K9). n=6, *P<0.01 at 0.1% serum; **P<0.01 at 0.1% and 10% serum.

K9+ BEN, BT-549, LX22 and 410.4 cells. No significant LDH release was detected in K9+ BEN or MDA-MB-231 cells (Fig. 4.6). This suggests that the observed reduction in cell viability could be attributed to increased cell death. The
correlation of cytotoxicity with target abundance is consistent with a KCNK9-specific mechanism.

**Discussion**

In this chapter, we demonstrated the utility of Y4 as a detection reagent of endogenous KCNK9 channels and as a tool for functional investigation of KCNK9 in cancer cells. Y4 is the first mAb generated to the extracellular domain of KCNK9 channel and the first specific inhibitor of KCNK9 function. Endogenous KCNK9 expression is often subdued under physiological conditions as high expression leads to strong K+ leak currents that cause cytotoxicity. The ability of Y4 to recognize endogenous channels is evidence for its superior affinity, and binding affinity of antibody modulators often correlates with target inhibition activity.

KCNK9 was found to be the only over-expressed gene within a novel chromosome 8q24.3 amplicon in human breast and lung cancers [22]. Enforced KCNK9 expression conferred tumorigenicity to otherwise nontumorigenic cells [22]. Since these initial studies, KCNK9’s oncogenic properties have been explored in multiple cancer cell lines [23-25]. Depending on the cancer type and method used to manipulate channel function, KCNK9 had different effects on tumorigenicity. For example, inhibiting KCNK9 via a dominant negative mutant, anti-sense shRNA or non-specific chemical blockers suppressed proliferation of lung carcinoma and melanoma cells [23, 24]. In contrast, pharmacological manipulation of KCNK9 with chemical modulators known to lack subtype specificity led to contradictory results in glioma cell lines [8]. Differences in these in vitro studies could be attributed to differences among cancer types or off-target effects of the chemical modulators. This further supports the need for highly selective pharmacological agents to
investigate KCNK9 function, and it highlights the advantage of mAb-based targeting.

**Experimental procedures**

**Survival analysis**

Kaplan-Meier curves for survival of patients with squamous cell lung cancer and breast cancer in relation to hKCNK9 gene expression level. Datasets were obtained from commonly used published studies [73, 74]. Patients’ gene expression data and clinical data were manually matched. Patients with both KCNK9 expression and survival data were segregated into two groups according to KCNK9 expression level – KCNK9 high (two-fold higher than mean) and KCNK9 low (two-fold lower than mean). Survival was analyzed using the Prism Kaplan Meier package and Log-rank Mantel-Cox statistical test. We performed the same analysis to examine hKCNK3 expression vs survival. n=53 (lung), n=289 (breast), *P<0.05.

**Cell viability assay**

Cancer cell lines were seeded in 10% FBS culture medium overnight at 37 °C. Next day, cells were washed with PBS and treated with 0.4 mg/ml antibodies in 0.1% or 10% FBS culture medium for 24 to 72 hours at 37 °C. Cell viability was measured using Cell Counting Kit-8/CCK8 (Dojindo Molecular Technologies). We added CCK substrate at 1/10 volume to cells and incubated at 37 °C for 2 hours. Colorimetric change was measured at 450 nm as determination of cell viability.

**Cell death assay**

Cell death was determined by measuring lactate dehydrogenase (LDH) release using CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to manufacturer’s instructions. Cancer cell lines were seeded in 10% FBS culture medium overnight at 37 °C. Next day, cells were washed with PBS and treated
with 0.4 mg/ml antibodies in 0.1% or 10% heat-inactivated FBS culture medium for 24 to 72 hours at 37 °C. Supernatant was collected to measure LDH released from dead cells (Abs490\text{dead}). We then added lysis buffer and collected supernatant to measure LDH released from live cells (Abs490\text{live}). Percent of cell death was calculated as: Abs490\text{dead}/(Abs490\text{dead}+ Abs490\text{live}).

**Cell cycle analysis**

KCNK9 channels on BEN cell surface were labeled with Y4 and secondary antibody as described in previous section. Cells were then fixed and permeablized with 80% ethanol followed by propidium iodide (PI) staining. During flow cytometry analysis, K9+ and K9- cells were gated and their corresponding PI signal was collected for analysis.

**Flow cytometry analysis of channel internalization**

BEN cells were incubated with 0.4 mg/ml Y-mAbs for 36 hours at 30 °C to induce endocytosis. KCNK9 channels remained on the plasma membrane were stained with a polyclonal anti-KCNK9 antibody followed by secondary antibody staining and flow-cytometry analysis as described above.

**QRT-PCR, western blot and flow cytometry**

Total RNA from cell lines and snap-frozen tumor tissues was extracted using RNeasy Mini Kit (Qiagen), and qRT–PCR was performed as described previously[76]. Relative expression of each gene was normalized to human18S rRNA or mouse GAPDH RNA. Sequences of primer pairs were listed in supplementary table 1.

Protein extraction and western blot were performed as described previously[76]. Single-cell suspensions were labeled with anti-KCNK9 mAbs for 30 minutes at 4 °C. After washing with ice-cold PBS, cells were incubated with APC-conjugated goat anti-mIgG secondary antibody for 30 minutes at 4 °C. Cell staining was analyzed
using FACS Calibur flow cytometer (BD Biosciences) or sorted using FACS Aria-II flow cytometer (BD Biosciences).
Chapter 5
Characterization of mAb Effects in vivo

Overview

KCNK9’s role in tumorigenicity has not been extensively explored in vivo. In one study, KCNK9 over-expression in mouse embryonic fibroblasts or mammary gland epithelial cells facilitates tumor formation in nude mice [23]. This oncogenic effect was found to depend on KCNK9 function, because enforced expression of a dominant negative KCNK9 mutant alone or in the presence of wild type KCNK9 abolished tumorigenicity. This in vivo study supports the notion that KCNK9 can support tumor development. However, it is based on KCNK9 over-expression, and up until now it had been unclear if targeting endogenous KCNK9 has any therapeutic benefits.

In this chapter, I will describe mAb-mediated targeting of KCNK9 in animal models and discuss the therapeutic potential KCNK9 antibody modulators.

Results

Assessment of mAb effects on human lung cancer xenografts

To investigate Y4’s therapeutic effects on tumor development, antibodies were administered intraperitoneally (i.p.) at 4 mg/kg into nude mice. We used two dosing regimens. In the first one, we treated animals with antibodies on the same day of BEN subcutaneous engraftments to monitor effects during tumor initiation. Under this regimen Y4 effectively suppressed tumor growth (Fig. 5.1a). In comparison, another KCNK9 mAb (H8) had no effect on tumor growth (Fig. 5.1c). Systemic administration of Y4 was well tolerated in mice as suggested by the absence of detectable effects on body weight or grooming (Fig. 5.1b). We also
Figure 5.1 Y4 inhibits BEN subcutaneous tumor growth
(a) Growth curves of BEN engraftments in nu/nu mice treated with mlG1 or Y4 i.p. twice a week for 22 days. n=10 per group, *P<0.01. Representative photographs showing tumors (dotted line) formed on day 22.
(b) Body weights of mice. n=10 per group.
(c) Growth curves of BEN engraftments in nu/nu mice treated with mlG1 or H8 i.p. twice a week for 25 days. n=10 per group.

Figure 5.2 Y4-treated tumors show reduced proliferative and apoptotic indices and KCNK9 level
(a) Quantitative determination of Ki67+ cells. n=30, *P<0.01.
(b) Quantitative determination of cleaved caspase-3+ cells. n=30, *P<0.01.
(c) Quantitative determination of KCNK9. n=10, *P<0.05.
Figure 5.3 Y4 inhibits development of established BEN subcutaneous tumors
(a) Growth curves of BEN engraftments in nu/nu mice treated with mlgG1 or Y4 i.p. twice a week for 14 days. n=10 per group, *P<0.01. Representative photographs showing tumors (dotted line) formed on day 14.
(b) Body weights of mice. n=10 per group.
(c) Quantitative determination of Ki67+ cells. n=30, *P<0.01.
(d) Quantitative determination of cleaved caspase-3+ cells. n=30, *P<0.01.
(e) Quantitative determination of KCNK9. n=10, *P<0.05.

Figure 5.4 Y4 inhibits the growth of LX22 patient-derived small cell lung cancer xenograft
(a) Growth curves of LX22 engraftments in NSG mice treated with mlgG1 or Y4 i.p. twice a week for 15 days. n=8 per group, *P<0.05, **P<0.01.
(b) Quantitative determination of Ki67+ cells. n=30.
(c) Quantitative determination of cleaved caspase-3+ cells. n=30.
(d) Quantitative determination of KCNK9. n=10.
examined molecular changes; Ki67 and cleaved caspase-3 staining showed that Y4-treated tumors had a lower proliferative index and a higher apoptotic index, respectively (Fig. 5.2a, b). Protein extractions from Y4-treated tumors also showed reduced KCNK9 expression normalized to β-actin (Fig. 5.2b). These results indicate that Y4 treatment hindered tumor growth by affecting proliferation and apoptosis, and it down-regulated tumor-associated KCNK9. In another dosing regimen, we treated animals only after visible tumors formed on day 8 to monitor effects on established tumors. This regimen is more clinically relevant. Y4 showed similar efficacy in inhibiting tumor formation with no apparent toxicity (Fig. 5.3a, b). We observed consistent changes in cellular markers and KCNK9 expression level (Fig. 5.3c).

Besides squamous cell lung cancer, KCNK9 over-expression has been reported in other types of lung cancer including small cell lung cancer for which effective therapies are lacking. Compared to xenografts derived from cancer cell lines, patient-derived xenografts (PDX) better represent human tumor heterogeneity and microenvironment making them ideal for drug efficacy and safety studies. Hence, we screened a number of small cell lung cancer PDX models and selected LX22 – a highly aggressive PDX model of small cell lung cancer [77] to verify the therapeutic potential of KCNK9-targeting via Y4. Since we did not observe significant differences between the two dosing regimens of BEN tumor treatment, we initiated Y4 treatment after visible LX22 tumors formed in NSG mice. LX22 PDX tumors showed larger variation compared to tumors formed of BEN cell line. Nevertheless, Y4 was effective on inhibiting LX22 tumor growth with no apparent toxicity (Fig. 5.4a). In addition, Y4-treated tumors had lower proliferative index as reflected by Ki-67 staining as well as KCNK9 down-regulation (Fig. 5.4b, d). This
further supports the notion that mAb-mediated targeting of KCNK9 can be a clinically valuable strategy for cancer treatment.

**Assessment of mAb effect on murine breast cancer cell metastasis**

Since we found relatively high KCNK9 expression in metastatic murine breast cancer cell lines, we went on to examine Y4’s effects on metastasis. The cross-reactivity of Y4 with mKCNK9 provided an opportunity to study in immunocompetent syngeneic animals. We injected 410.4 cells into the tail vein of BALB/cByJ mice and examined Y4’s effects on lung colonization. Y4-treated animals showed significantly fewer lung metastases with no detectable toxicity (Fig. 5.5). These data suggest that Y4 not only is effective against primary tumor formation but also metastasis.

**Discussion**

Within this chapter, we demonstrated the therapeutic potential of Y4, and mAb-based KCNK9 targeting in general. Y4 is the first reported ion-channel targeting antibody that has effective anti-cancer activity in vivo. We obtained consistent effects of Y4 on tumor growth and metastasis, supporting the importance of specificity in functional manipulation as discussed in Chapter 4.

Numerous K+ channels in addition to KCNK9 have been reported to be differentially expressed in human cancer and regulate different aspects of tumorigenicity [78, 79]. However, it is still largely unknown how K+ ion conductance controls biological processes. K+ channels, K2P in particular, fine
tune resting membrane potential to keep it at a generally defined range of -30 to -85 mV. As channels open, the membrane potential can either become more negative (hyperpolarize), or become more positive (depolarize) in comparison to the resting membrane potential. Changes in membrane potential can alter cell physiology such as cell volume dynamics that in turn regulate cell proliferation, adhesion and migration [78, 79]. For example, calcium (Ca\(^{2+}\)) -activated K\(^{+}\) channels (KCa) and inward rectifying K\(^{+}\) channels (Kir) can regulate local cell volume to promote cancer cell invasion [78]. In addition, maintenance of resting membrane potential is critical to the function of voltage-sensitive molecules. For example, hyperpolarization caused by opening of K2P channels increases Ca\(^{2+}\) influx and regulates G1-S transition during cell cycle progression [80]. Membrane potential oscillation may also directly control cell cycle progression. The expression and function of K+ channels are known to show cell cycle phase-dependence [78]. A number of reports indicate that membrane hyperpolarization accompanied with increased K\(^{+}\) permeability takes place at G1-S transition and it is required to initiate S phase, whereas depolarization prevents G1-S transition. Preliminary cell cycle analysis with BEN cells indicated that KCNK9 expression disproportionally associated with S+G2/M phases. Since the opening of KCNK9 results in hyperpolarization, it suggests that increased expression/activity of KCNK9 promotes cell cycle progression through G1-S. This would support the findings that KCNK9 targeting by Y4 reduced proliferative index and decreased tumor burden in vivo. In our study, by comparing Y4 and H8, we found that the degree of KCNK9 conductance inhibition correlates with antibody’s anti-cancer effect in vivo. Also, modulation of KCNK9 by Y4 in the absence of immune-dependent factors such as complement is sufficient to cause profound cytotoxicity in vitro. These results
suggest that KCNK9 is not simply a tumor antigen but an important regulator of tumor growth. Defining the mechanistic network linking K+ homeostasis and tumor physiology awaits further studies.

**Experimental procedures**

**Xenograft studies**

Female 6-to 8-week-old nu/nu or NSG mice (Charles River) were anesthetized by intraperitoneal (i.p.) injection of 5 mg/kg xylazine. Mice received 4×10^6 viable BEN cells in 25 µL PBS and 25 µL Matrigel (Corning) subcutaneously (s.c.) or 0.5×10^6 viable LX22 cells in 50 µL PBS and 50 µL Matrigel (Corning) s.c. in the dorsal flank. To monitor mAbs’ effects on tumor initiation, mice were randomly divided into two groups and received 4 mg/kg mIgG1 or Y4 in 100 µL PBS i.p. starting on day 0 followed by Ab injection twice a week. To monitor mAbs’ effects on tumor regression, after tumors reached 50 mm³, mice were randomly divided into two groups and received 4 mg/kg mIgG1 or Y4 in 100 µL PBS i.p. twice a week. Tumor volumes were estimated by measuring two dimensions [length (a) and width (b)] and calculated using the equation: V = ab²/2 as described previously [76]. All mice were used in accordance with guidelines from the Johns Hopkins School of Medicine Animal Care Committee.

**Metastasis studies**

Female 4-to 6-week-old Balb/cByJ mice (Jackson Laboratory) were randomly divided into two groups and primed by receiving 4 mg/kg mIgG1 or Y4 in 100 µL PBS i.p. twice a week for one week prior to cell injection. 2×10^5 viable 410.4 cells were injected i.v. into the lateral tail vein of mice which were then treated with 4 mg/kg mIgG1 or Y4 in 100 µL PBS i.p. twice a week. All mice were euthanized on day 25 post-transplantation or earlier if moribund. Lungs were examined for
surface tumor colonies under a dissecting microscope. All mice were used in accordance with guidelines from the University of Maryland Institutional Animal Care Committee.

**Immunohistochemistry**

Cryostat sections were stained with anti-cleaved caspase-3 and anti-Ki67 antibodies and counterstained with Methyl Green as previously described [81]. Proliferation and apoptotic indices were determined by computer-assisted quantification using ImageJ Software (rsb.info.nih.gov/ij/) as previously reported [81].
Chapter 6
Characterization of Immune-dependent Effects of mAbs

Overview

Murine mIgG1 is capable of eliciting effector responses via the Fc domain, including complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). These responses can facilitate the targeting of KCNK9-expressing tumor cells by recruiting and/or activating immune components.

In previous chapters, we focused on the cell-autonomous effects of KCNK9 antibodies and show that modulation of KCNK9 function increases cytotoxicity. In this chapter, I will describe immune-dependent targeting of KCNK9 via antibodies.

Results

Induction of immune markers

Y4 is murine IgG1 isotype which possesses the ability to mediate effector functions. Thus, inhibition of lung metastasis in immune-competent hosts could be due to direct effects via KCNK9 inhibition or elevated immune responses via mAb effector functions. We extracted mRNA from lung tissues and characterized the expression of immune markers [82]. Y4-treated mice showed higher infiltration

Figure 6.1 Y4 induces markers of immune responsiveness
Expression of immune markers verified by qRT-PCR of mRNAs extracted from lung tissues. n=5, *P<0.05.
of CD56+ natural killer (NK) cells, granzyme B+ cytotoxic T cells as well as increased T cell chemoattractant CXCL-9 (Fig. 6b). We have shown previously that enforced expression of CXCL9 inhibits growth and metastasis of murine breast cancer cells by a mechanism involving NK and T cells [82]. This suggests that Y4-mediated inhibition of metastasis could involve induction of immune cell infiltration and possibly ADCC.

**Activation of complement-dependent cytotoxicity**

To investigate if Y4 can elicit complement-dependent CDC, we incubated tumor cells with Y4 for 2 hours in the presence of murine complement prior to LDH assay. Y4 was effective in inducing CDC against K9+ BEN and BT-549 cells. This effector response was dependent on the complement concentration, while no effects were found in K9- BEN cells or if complement was heat-inactivated (Fig. 4.8). Hence, besides direct killing of cancer cells via KCNK9 modulation, Y4 is capable of inducing CDC of KCNK9-expressing cells.

![Figure 6.2](image_url)

**Figure 6.2 Y4 activates complement, resulting in complement-dependent cytotoxicity in vitro**

K9+ BEN cells and BT-549 cells were treated with normal or heat inactivated murine complement before LDH measurement. n=6, *P<0.05.

**Activation of antibody-dependent cellular cytotoxicity**

To investigate the possibility of Y4-induced ADCC, we incubated BEN cells with increasing Y4 concentrations in the presence of bioluminescent Jurkat T
reporter cells established for ADCC studies [83] (Promega). We found a dose-dependent induction of ADCC as measured by luciferase activity. Maximal induction was 29-fold with an EC50 at 0.4 µg/ml (Fig. 6.3). Since Y4 is capable of eliciting effector responses in vitro, it is plausible that Y4-mediated inhibition of metastasis is attributed to a combination of immune-independent and immune-dependent mechanisms.

![Figure 6.3 Y4 activates ADCC reporter cells](image)

<table>
<thead>
<tr>
<th></th>
<th>EC50, ng/ml</th>
<th>Induction, fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y4</td>
<td>415 ± 36</td>
<td>29 ± 0.4</td>
</tr>
<tr>
<td>mlgG1</td>
<td>1121 ± 805</td>
<td>4 ± 0.5</td>
</tr>
</tbody>
</table>

**Figure 6.3 Y4 activates ADCC reporter cells**

BEN cells were incubated with different doses of Y4 and mlgG1, and engineered murine Jurkat T cells stably expressing FCγRIII and an NFAT-response element that drives luciferase expression (NFAT-RE-luc2). Measured luciferase activity indicates ADCC induction. n=3, *P<0.01.

**Discussion**

We have discussed in Chapter 3 that Y4-induced anti-cancer activity can involve multiple pathways. Y4 antibodies can have anti-cancer effects independent of KCNK9 function. For instance, antibody Fc domain is associated with effector functions. Effector cells with Fc receptors (FcRs) on the cell surface are able to interact with antibody-coated cells, mediating ADCC. Moreover, Fc region can
directly stimulate complement activation, leading to CDC. As a result, target cells are either destroyed by phagocytosis or lysis. Indeed, we show that Y4 is capable of eliciting ADCC and CDC. Both cell autonomous and immune-dependent properties of Y4 can be further explored to improve its therapeutic value via chemical modification of specific domains. For example, the efficiency of triggering immune events varies for different IgG subtypes. Murine IgG2a and human IgG1 have the strongest ADCC activity. Switching the constant domain of Y4 with these isotypes can enhance the targeting of cancer cells via ADCC. The versatile mechanisms of antibody-based targeting and the flexibility afforded by antibody engineering highlight the advantages of antibody-based targeting compared to conventional modulation by small molecules.

Experimental procedures

QRT-PCR

Total RNA from snap-frozen lung tissues was extracted using RNeasy Mini Kit (Qiagen), and qRT-PCR was performed as described previously [76]. Relative expression of each gene was normalized to mouse GAPDH RNA. Sequences of primer pairs were listed in supplementary table 1.

Complement-dependent cytotoxicity (CDC)

CDC assay protocol was adapted from previous studies [84, 85]. Cancer cell lines were treated with 0.4 mg/ml antibodies with or without murine serum complement (Rockland) for 2 hours at 37 °C. Lyophilized complement sera from mouse was reconstituted in PBS and serially diluted 1:10 to 1:1000. Heat-inactivation was performed by 30-minute incubation at 56 °C. Cell death was determined as LDH release.

Antibody-dependent cellular cytotoxicity (ADCC) reporter assay
ADCC assay was performed following manufacturer’s recommendations [83]. Briefly, BEN target cells were seeded the day before the assay. Y4 and mIgG1 were titrated with a maximal concentration of 100 µg/ml, added to BEN cells and incubated for 30 minutes at 37 °C. Murine Jurkat NFAT-luc+FcγRIIIa cell line was used as effector cells and seeded at a ratio of Jurkat:BEN = 6:1. Co-culture was incubated for 6 hours at 37 °C. Luciferase induction was measured as a readout of ADCC using Bio-Glo Luciferase assay reagent and a Tecan plate reader.
Chapter 7

Summary and Future Directions

Summary

In this work, we described the development of monoclonal antibodies targeted to the extracellular domain of the KCNK9 channel and the extensive characterization of their biochemical, cell biologic and pre-clinical therapeutic properties in solid tumor models. Among all the antibodies generated, Y4 appears to be the most promising candidate. It demonstrates high affinity and binding specificity (Chapter 2). Addition of Y4 to KCNK9-expressing cells inhibits channel conductance as a result of antibody-induced channel internalization (Chapter 3). At the cellular level, Y4 inhibits cell viability and increases cell death in KCNK9-expressing cancer cells (Chapter 4). Systemic administration of Y4 effectively inhibits the growth of human lung cancer xenografts and murine breast cancer metastasis with no apparent toxicity (Chapter 5). Additionally, Y4 has the capacity to elicit immune-dependent anti-cancer activity as evidenced by increased infiltration of immune cells into Y4-treated lung tissues as well as activation of complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity (Chapter 6). Our study serves as a proof of principle for antibody-based targeting of ion channels. Monoclonal antibodies reported here are the first to target the extracellular domain of KCNK9 channel, and Y4 is the first ion channel-targeting monoclonal antibody that is efficacious in treating malignancies in vivo.

Future Directions

Our study with Y4 significantly advances our understanding of KCNK9’s role in cancer and the clinical significance of antibody-based targeting of KCNK9. For further therapeutic development, we would like to take Y4 as a prototype and
generate humanized derivatives. Y4’s ability to kill tumor cells involves both cell-autonomous and immune-dependent mechanisms. The former relies mainly on high affinity interaction between the antigen-binding regions of Y4 and KCNK9. Immune effector functions are determined by the interaction between Y4’s constant regions (Fc) and complement complexes or Fc receptors on immune cells. Besides humanization, chemical modifications within Y4’s functional regions could improve its binding and effector functions [29, 30]. For instance, we can switch mIgG1 Fc with hIgG1 Fc. hIgG1 has most potent ADCC activity and we expect to see more robust anti-cancer immune responses.

Once we have humanized Y4 and validated its activity in existing assays, we can further explore its therapeutic properties. One important question we would like to address is how Y4 fits within the current cancer treatment paradigm. We can perform in vitro and in vivo assays combining Y4 with currently available therapies including cytotoxic agents, radiation and checkpoint inhibitors. Results from these studies will provide a better understanding of Y4’ therapeutic potential in comparison to existing strategies and if synergism with other treatment modalities are possible. In addition, humanized Y4 can be further engineered as diagnostics or antibody conjugates. By tagging Y4 with a cytotoxic or radioactive payload, one can achieve specific and effective killing of KCNK9-positive tumor cells.

In sum, Y4 represents a novel and imminently clinically translatable inhibitor of a novel cell surface cancer target. Our study paves the road for future therapeutic targeting of KCNK9 and other ion channels involved in malignancies and potentially other disorders.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Ion channel target</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Lambert-Eaton Syndrome        | P/Q-, N-Ca\textsubscript{\alpha} | Internalize and reduce cell surface channels  
 Reduce presynaptic Ca\textsuperscript{2+} influx  
 Reduce acetylcholine release | [44]   |
| Neuromyotonia                 | K\textsubscript{\alpha}1 complex   | Internalize and reduce cell surface channels  
 Reduce presynaptic K\textsuperscript{+} influx  
 Increase acetylcholine release | [86]   |
| Limbic encephalitis           | K\textsubscript{\alpha}1 complex   | Internalize and reduce cell surface channels  
 Reduce presynaptic K\textsuperscript{+} influx  
 Increase acetylcholine release | [86]   |
| Morvan’s syndrome             | K\textsubscript{\alpha}1 complex   | Internalize and reduce cell surface channels  
 Reduce presynaptic K\textsuperscript{+} influx  
 Increase acetylcholine release | [86]   |
| Dilated cardiomyopathy        | KCNQ1               | Increase IK\textsubscript{s} current     | [44]   |
| Myasthenia gravis             | nAChR               | Internalize and reduce cell surface channels  
 Interfere ligand binding | [44]   |
| Cerebellar ataxia             | mGluR 1\alpha       | Internalize and reduce cell surface channels | [87]   |
| Rasmussen’s encephalitis      | α7 nAChR            | Reduce channel conductance              | [88]   |
Table 3 Summary of engineered antibodies targeting voltage-sensitive ion channels

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<th>Channel family</th>
<th>Antibody</th>
<th>Region</th>
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<th>Cell type</th>
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<td>E3 extracellular loop in domain I</td>
<td>FAEADERDSQFPSIP</td>
<td>HEK-293 NG108-15</td>
<td>Reduce whole cell current; Restore consciousness during anesthesia</td>
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<td>Central medial thalamus (rat)</td>
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<td>Primary oligodendrocyte progenitor cell (mouse)</td>
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<td>GSGSGKWEG</td>
<td>HEK-293 Xenopus oocytes Neuroblastoma</td>
<td>Reduce whole cell current; Reduce tumor growth</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Acute myeloid leukemia</td>
<td>HEL, UT-7, K562, PLB-985</td>
<td>Reduce whole cell current; reduce action potential amplitude; reduce Vmax; reduce membrane responsiveness</td>
<td>[37, 40]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Voltage-gated sodium channels**

<table>
<thead>
<tr>
<th>Voltage-gated sodium channels</th>
<th>Anti-Nav (SC-72-14)</th>
<th>Extracellular domain</th>
<th>N/A</th>
</tr>
</thead>
</table>

<p>| Sciatic nerve fibers (rat)    | Reduce whole cell current; reduce action potential amplitude; reduce Vmax; reduce membrane responsiveness | [37, 40] |
| Optic nerve fibers (rat)      |                                                                                                 |           |
| Cardiac Purkinje fibers (canine) |                                                                                      | [40]     |
| Sciatic nerve fibers (rat)    | Reduce whole cell current;                                                                 | [39]     |</p>
<table>
<thead>
<tr>
<th>Antibody/Protein</th>
<th>Extracellular Domain</th>
<th>Antigen</th>
<th>Action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Na&lt;sub&gt;v&lt;/sub&gt; (SC-72-38)</td>
<td>Extracellular domain</td>
<td>N/A</td>
<td>Shift the voltage dependence of activation and inactivation</td>
<td>[41]</td>
</tr>
<tr>
<td>Anti-Na&lt;sub&gt;v&lt;/sub&gt; (SC-66-5)</td>
<td>Extracellular domain</td>
<td>N/A</td>
<td>Induce channel internalization</td>
<td>[38]</td>
</tr>
<tr>
<td>Anti-Na&lt;sub&gt;v&lt;/sub&gt; 1.5</td>
<td>E3 extracellular loop in domain I</td>
<td>CVRNFTALNGTNGSV EAD</td>
<td>Reduce whole cell current</td>
<td>[46]</td>
</tr>
<tr>
<td>Anti-Na&lt;sub&gt;v&lt;/sub&gt; 1.5</td>
<td>E2 extracellular loop in domain I</td>
<td>VSENIKLGNLSALRC</td>
<td>Reduce whole cell current</td>
<td>[55]</td>
</tr>
<tr>
<td>Anti-L-type</td>
<td>Extracellular domain</td>
<td>N/A</td>
<td>Reduce slow current</td>
<td>[42]</td>
</tr>
<tr>
<td>Anti-α&lt;sub&gt;1D&lt;/sub&gt;</td>
<td>C-terminal to the pore-forming region between S1 and S2 in domain IV</td>
<td>KLCDPDSYNPGEEY TC</td>
<td>Reduce L-type current (use dependent)</td>
<td>[91]</td>
</tr>
</tbody>
</table>

**Voltage-gated calcium channels**
<table>
<thead>
<tr>
<th>Anti-N and P/Q type</th>
<th>E3 extracellular loop</th>
<th>DESKEFERDCRGK</th>
<th>Cerebellar granule neurons (mouse)</th>
<th>Reduce N-type current, P/Q-type current, excitatory postsynaptic current</th>
<th>Induce cerebellar ataxia phenotype</th>
<th>[48]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-P-type</td>
<td>E3 extracellular loop</td>
<td>IDVEDEDSDEDEFC</td>
<td>Small-cell lung carcinoma</td>
<td>Reduce P-type current</td>
<td></td>
<td>[92]</td>
</tr>
<tr>
<td>TRPC1</td>
<td>E3 extracellular loop</td>
<td>QLYDKGYTSKEQKDC</td>
<td>Platelets and vascular endothelial cells (human)</td>
<td>Reduce agonist-evoked or store-operated calcium entry</td>
<td></td>
<td>[50, 88]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CVGIFCEQQSNDTFH SFIGT</td>
<td>Vascular smooth muscle cells (human)</td>
<td>Reduce store-operated calcium entry</td>
<td></td>
<td>[49]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bovine aortic endothelial cells</td>
<td>Reduce store-independent, agonist-evoked calcium entry</td>
<td></td>
<td>[93]</td>
</tr>
<tr>
<td>Anti-TRPC5</td>
<td>E3 extracellular loop</td>
<td>CYETRAIDEPNNCKG</td>
<td>HEK293 CHO Cerebral arterioles (rabbit) Pial arterioles (rabbit)</td>
<td>Reduce L-type current</td>
<td></td>
<td>[46, 94]</td>
</tr>
<tr>
<td>Anti-TRPM3</td>
<td>E3 extracellular loop</td>
<td>CLFPNEEPSWKLAN</td>
<td>HEK293</td>
<td>Reduce whole cell current [95]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>----------------------</td>
<td>----------------</td>
<td>--------</td>
<td>-------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-TRPV1</td>
<td>E3 extracellular loop</td>
<td>EDGKNNSLPMESTPH</td>
<td>CHO HEK293</td>
<td>Reduce channel activation by proton, heat and chemical ligands [96]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 4. QRT-PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>hKCNK9</td>
<td>NM_016601</td>
<td>5'-GCTCCTTCTACTTTTGCATACG-3’</td>
<td>5'-CTGGAACATGACCAGTGCAG-3’</td>
</tr>
<tr>
<td>mKCNK9</td>
<td>NM_001033876</td>
<td>5'-CCCCTGAGCTGTGTTATGTC-3’</td>
<td>5'-CATGCCACAGCACTTTCTTG-3’</td>
</tr>
<tr>
<td>mCD4</td>
<td>NM_013488</td>
<td>5'-ACACACCTGTGCAAGAAGCA-3’</td>
<td>5'-GCTCTTGGTTGGGAGAATC-3’</td>
</tr>
<tr>
<td>mCD8</td>
<td>NM_009856</td>
<td>5'-CTCACCTGTGCACCCTACC-3’</td>
<td>5'-ATCCGGTCCCCTTCAGT-3’</td>
</tr>
<tr>
<td>mCD20</td>
<td>NM_026956</td>
<td>5'-AACCTGCTCCAAAGTGAGG-3’</td>
<td>5'-CCCAGGGAATATGGAAGAGGC-3’</td>
</tr>
<tr>
<td>mCD25</td>
<td>NM_008367</td>
<td>5'-GCGTTTGGCTTGAAGAATCTCCTGG-3’</td>
<td>5'-GCATAGACTGTTGGGCTTGC-3’</td>
</tr>
<tr>
<td>mCD56</td>
<td>NM_001081445</td>
<td>5'-GGTTCAGGAATGGTCAGTTG-3’</td>
<td>5'-CAAGGACTCTCTGGCAGTGACG-3’</td>
</tr>
<tr>
<td>mCD68</td>
<td>NM_009853</td>
<td>5'-CCATCCTTCACGACAGACCT-3’</td>
<td>5'-GGCAGGGTTATGAGTGACAGT-3’</td>
</tr>
<tr>
<td>mCD279</td>
<td>NM_008798</td>
<td>5'-CGGTTTGAAGGATGGTCATTGG-3’</td>
<td>5'-TCAGAGTGCCTCTGCTTCC-3’</td>
</tr>
<tr>
<td>mGranzyme B</td>
<td>NM_013542</td>
<td>5'-CCACTCTTCAGCCCTACATGG-3’</td>
<td>5'-GGCCCCAAAGTGACATTTATT-3’</td>
</tr>
<tr>
<td>mCXCL9</td>
<td>NM_008599</td>
<td>5'-CCTAGTGATAAGGATGCAGAG-3’</td>
<td>5'-CTAGGGAGTTGTAGCTCCGTTT-3’</td>
</tr>
<tr>
<td>mCXCL11</td>
<td>NM_019494</td>
<td>5'-GGCTTTTTGTTGGAGAAGGG-3’</td>
<td>5'-GCGTTACTCGGGAATTACA-3’</td>
</tr>
<tr>
<td>h18s rRNA</td>
<td>X03205</td>
<td>5'-GTAACCCGTGAAACCCATT-3’</td>
<td>5'-CCATCAAGTGGTAGTAGCG-3’</td>
</tr>
<tr>
<td>mGAPDH</td>
<td>NM_008084</td>
<td>5'-CATCAGTGCACCCAGAAGACT-3’</td>
<td>5'-ATGCAAGTGCCTCCGTTCA-3’</td>
</tr>
</tbody>
</table>
References


Han Sun
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Education

The Johns Hopkins University, School of Medicine
Ph.D. in Cellular and Molecular Medicine  Baltimore, MD  Jul. 2010-Oct. 2015

Nanyang Technological University
Bachelor of Science with Honors in Biological Sciences  Singapore  Jul. 2004-Jul. 2008

Professional Experience

The Johns Hopkins University, School of Medicine

Advisors: Dr. Min Li and Dr. John Laterra  Jul. 2011-Oct. 2015

Doctoral thesis research: “Development of Functional Antibodies to Probe Two Pore Potassium Channels in Cancer”

• Designed antigen and immunization strategy to generate the first subtype-specific monoclonal antibody targeting the extracellular domain of KCNK9 ion channel
• Developed high throughput biochemical and functional assays for screening of antibody candidates
• Identified endocytosis as the mechanism of action of antibody-mediated channel inhibition
• Characterized antibodies’ cytotoxic effects and effector functions in multiple cancer cell lines
• Utilized 3 different animal models to characterize antibodies’ effects on primary tumor growth and metastasis
• Filed 1 patent application
• Published 1 review article and prepared 1 manuscript currently under review
• Mentored post-doctoral fellow, graduate rotation students, and technicians to generate recombinant proteins for antibody development or biochemistry studies
• Managed collaboration with 7 academic laboratories

The Johns Hopkins University, School of Medicine

Advisors: Dr. John Laterra and Dr. Mingyao, Ying  Jun. 2014-Oct. 2015

Graduate research: “Repurposing Small Molecule Drug Verteporfin to Inhibit TAZ/YAP-driven Signaling and Tumorigenicity of Cancer Cells”

• Characterized the cytotoxic effects and mechanism of action of verteporfin in cell line models of triple-negative breast cancer, cancer stem cell models of human glioblastoma and medulloblastoma, and primary cultures derived from patient-derived glioblastoma xenograft
• Developed a micelle-based formulation which improves the bioavailability of verteporfin and enables in vivo imaging of drug delivery
• Utilized ultra performance liquid chromatography combined with mass spectrometry to quantify the distribution, metabolism and elimination of verteporfin in vivo
• Characterized the therapeutic benefits of verteporfin in human breast cancer subcutaneous xenograft and human breast cancer orthotopic xenograft
• Identified novel gene signature and signaling pathways that mediate verteporfin responses
• Prepared 1 grant proposal submitted to the Department of Defense Breast Cancer Research Program
• Prepared 1 manuscript in the process of submission
• Managed collaboration with 2 academic laboratories

The Johns Hopkins University, School of Medicine
Graduate rotation research: “Promoter Methylation of Genes Associated with Methotrexate Resistance in Leukemia and Lung Carcinoma”
• Screened 88 clinical samples of patients with leukemia or lung carcinoma by methylation-specific PCR and bisulfite sequencing
• Identified promoter methylation and decreased expression of proton-coupled folate transporter and CTP synthase which may determine sensitivity to methotrexate therapies

The Johns Hopkins University, School of Medicine
Graduate rotation research: “Characterization of Endogenous Retroviral Repeats in Primary Lung Tumors as Potential Immunotherapy Targets”
• Optimized extraction of high quality RNA (RNA Integrity Number>5 and 28S:18S rRNA>7) from formalin-fixed paraffin embedded tissues for array analysis
• Identified families of endogenous retroviral repeats differentially expressed in lung tumors as potential targets of cancer vaccines

The Johns Hopkins University, School of Medicine
Advisor: Dr. Min Li Jul. 2010–Dec. 2010
Graduate rotation research on “Expression and Purification of KCNK9 Extracellular Domain for Antibody Generation”
• Established large-scale mammalian expression platform of recombinant KCNK9 proteins
• Developed a protocol for effective purification of high quality recombinant KCNK9 proteins
• Initiated and managed collaboration with 2 academic laboratories

The Johns Hopkins University, School of Medicine
Bachelor thesis research on “Distinct Roles of LynA and LynB Kinases in Antigen-induced MAP Kinase Activation and Mast Cell Spreading”
• Discovered the role of membrane compartmentalization in regulating Lyn kinase activity
• Identified differential regulation of cell spreading by two Lyn kinase isoforms in primary murine mast cells

Nanyang Technological University, School of Biological Sciences
Undergraduate research on “Molecular Mechanism and Three-dimensional Structural Studies of Immunosuppressant FK-506 Binding Protein 38”
• Generated high-quality recombinant FK-506 binding protein 38 for NMR studies

Publications

Review article
• Han Sun and Min Li, “Antibody therapeutics targeting ion channels: are we there yet?”, Acta Pharmacologica Sinica, 2013, volume 34: 199–204.
Research articles
• Han Sun, Liqun Luo, Bachchu Lal, Xinrong Ma, Lieping Chen, Christine L. Hann, Amy Fulton, Daniel Leahy, John Laterra, and Min Li., “A Novel Monoclonal Antibody Against KCNK9 K+ Channel Extracellular Domain Inhibits Carcinoma Cell Viability, Tumor Growth and Metastasis”. (under review)
• Han Sun, John Laterra, and Mingyao Ying, “Repurposing Small Molecule Drug Verteporfin to Inhibit TAZ/YAP-driven Signaling and Tumorigenicity of Breast Cancer Cells”. (manuscript in preparation)

Conference proceedings
• Han Sun, Liqun Luo, Bachchu Lal, John Laterra, and Min Li, “Probing the Tumorigenic Properties of Two Pore Potassium Channels using Inhibitory KCNK9 mAbs”, 2015 PEGS: the essential protein engineering summit, May 4-8 2015, Boston, MA USA.
• Han Sun and Min Li, “Magic Bullets to Ion Channels – Generate Monoclonal Antibodies to Stop the “Leak” in Cancer Cells”, 2012 HIT Center Symposium on Human Systems Biology, Nov 15 2012, Baltimore MD, USA.

Invited Talks
• Han Sun and Min Li, “Antibody therapeutics targeting ion channels: are we there yet?”, 2013 Discovery on Target, September 24-25 2013, Boston MA, USA.
• Han Sun, Liqun Luo, Bachchu Lal, John Laterra, and Min Li, “Probing the Function of Two Pore Potassium Channels – Development of Active Antibodies”, University of Maryland Department of Pathology, September 26 2014, Baltimore MD, USA.

Patent

Awards
Graduate fellowship awarded by JHMI Jul. 2010-Present
<table>
<thead>
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<th>Professional Membership</th>
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<tbody>
<tr>
<td>American Association for Cancer Research Membership</td>
<td>2014-Present</td>
</tr>
<tr>
<td>Women in Cancer Research Membership</td>
<td>2015-Present</td>
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</table>