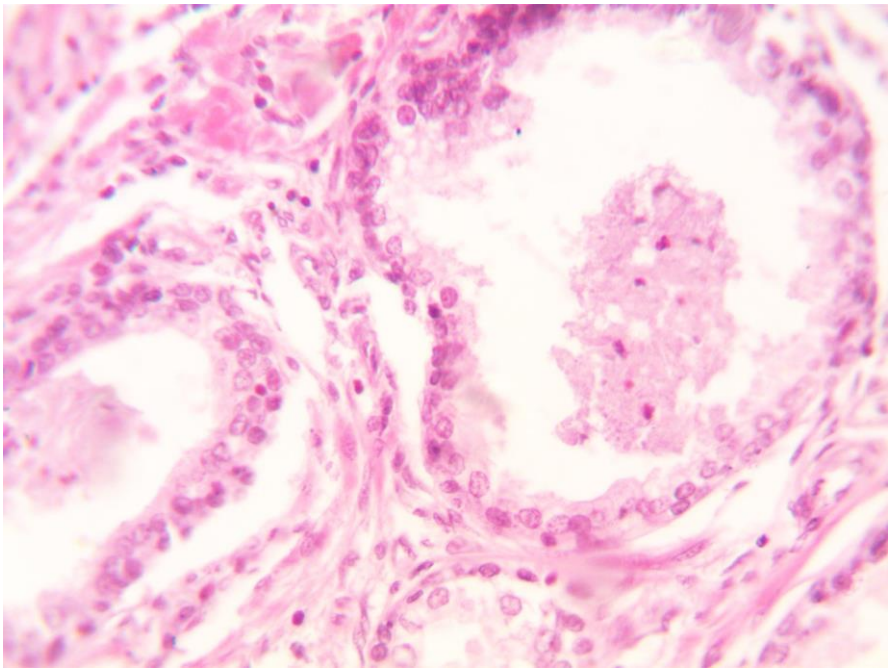

PSMA: A PROSTATE CANCER TARGET

Terrence P. McGarty

ABSTRACT
PSMA is a surface protein which is highly prevalent on metastatic prostate cancer cells. We use this example as a paradigm to consider tumor specific markers and a multiplicity of therapeutic options.

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Contents

1 INTRODUCTION

One of the factors that have arisen when addressing cancer treatments is the ability to identify uniquely the malignant cell as compared to the benign cell. We consider here the issue of prostate cancer and the prevalence of a specific and somewhat unique surface protein, PSMA, which is most often found on the surface of malignant cells. We know that this protein enhances the multiple internal pathways in the malignant cells making them proliferate and enhance metastasis. We demonstrate below a somewhat simplified view of this issue. We know the following:

1. Internal pathways often lead to the many characteristics of metastatic behaviour. Such gene products as p53, PTEN, mTOR, Akt can be activated or deactivated and the result in a metastatic cancer.

2. Surface markers which are unique can assist in targeting the specific cells involved in malignancy.

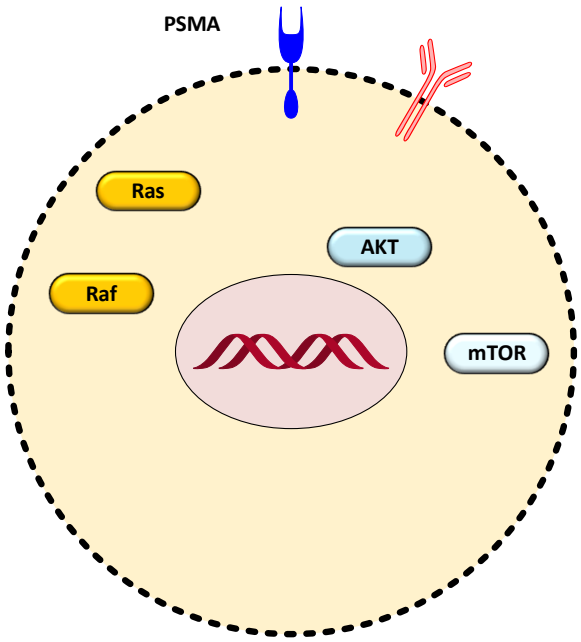
3. There now are a set of "tools" which allow the targeting of the unique markers while at the same time impacting the internal cellular dynamics.

4. However, and this is critical, many solid tumors can be "protected" by the tumor micro-environment, TME, of fibroblasts, macrophages, and other such supportive stroma cells.

Thus, it appears that having the specific targeting is critical, necessary but not sufficient.

In this report we examine a target for prostate cancer, PCa, and then examine ow we may apply the multiplicity of techniques to address the cancer.

The graphic below demonstrates some of the approaches to dealing with malignant cells. First one must be able to identify them often via some unique surface marker such as PSMA. Second one must understand what role the marker plays in the internal workings of the cell so as to effect its malignant sate. Third one must be able to assess what therapeutic strategies can be employed.



Now this is one of many such proteins but it can be used as an example of how a multiplicity of targeting of the malignant cells can be performed. We use this surface marker and its internal channels to consider a multiplicity of therapeutic targets.

We now look at the methods by which we can use this type of marker and address a set of therapeutic approaches.

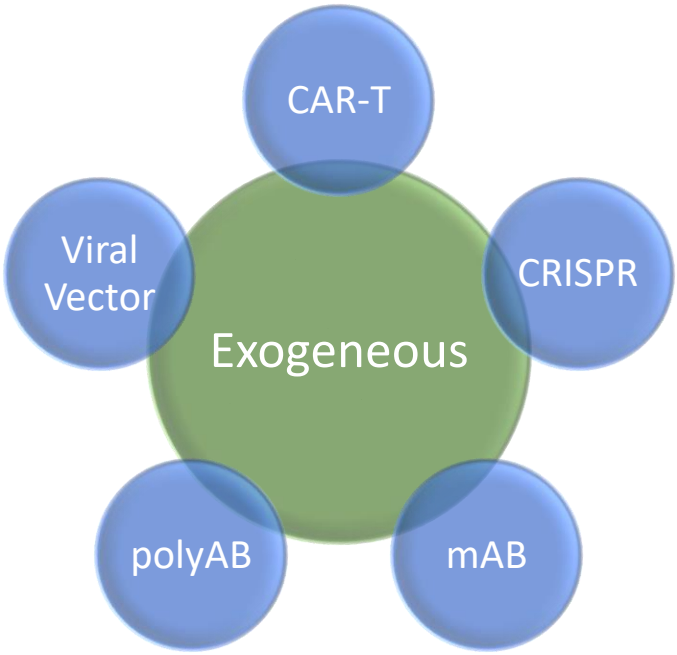
1.1 EXOGENEOUS

Exogenous approaches are means and methods we have in the tool box to use the specific marker and then have some external approach to attacking the malignant cell.

As Handa et al note:

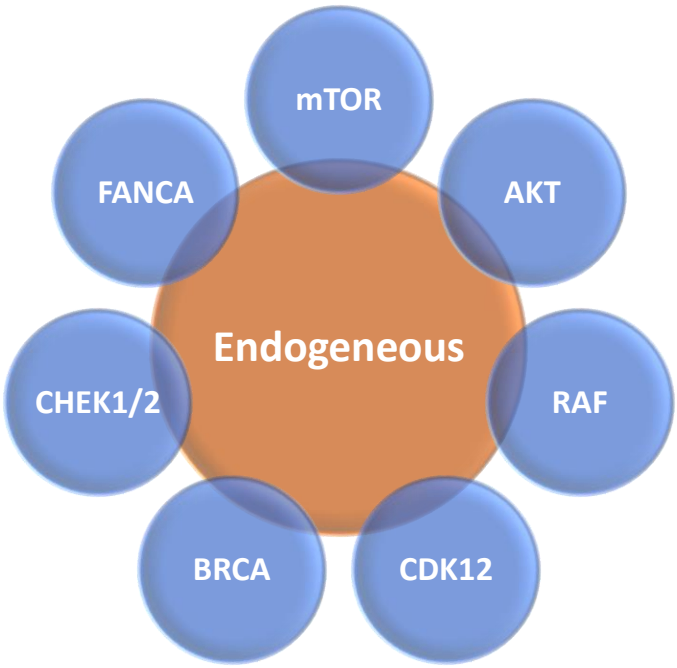
Metastatic castrate resistant prostate cancer (PCa) remains an incurable entity. In the era of immunotherapy, the complex PCa microenvironment poses a unique challenge to the successful application of this class of agents. However, in the last decade, a tremendous effort has been made to explore this field of therapeutics.

In this review, the physiology of the cancer immunity cycle is highlighted in the context of the prostate tumor microenvironment, and the current evidence for use of various classes of immunotherapy agents including vaccines (dendritic cell based, viral vector based and DNA/mRNA based), immune checkpoint inhibitors, Chimeric antigen receptor T cell therapy, antibody-mediated radioimmunotherapy, antibody drug conjugates, and bispecific antibodies, is consolidated.



1.2 ENDOGENOUS

The endogenous approach focuses on the internal mechanisms and pathways that we know are in malignant cells to stop the aberrant ones. Imatinib is a now classic example used in CML. There now are hundreds of ways that these have been used. We show some examples below.



Some gene targets[1] from various trials are as follows:

1.2.1 TRITON Trial

In this trial the following genes were targeted:

BRCA, PALB2, FANCA, BRIP1, and RAD51B. ATM, CHEK2

As has been noted the TRITON Trial is as follows[2]:

The pivotal findings from the phase 2 TRITON2 trial, which led to the FDA approval of rucaparib (Rubraca) in BRCA-positive metastatic castration-resistant prostate cancer (mCRPC), have now been published online in the Journal of Clinical Oncology.

In the study, the PARP inhibitor[3] induced a confirmed objective response rate (ORR) per independent review of 43.5% in heavily pretreated patients with mCRPC and a deleterious BRCA alteration.

The ORRs were similar, regardless of whether patients had germline or somatic alterations, or whether the alteration was BRCA1 or BRCA2.

The investigators did report, however, that PSA response rate was higher among patients with BRCA2 alterations. Based on the TRITON2 data, the FDA approved rucaparib in May 2020 for the treatment of patients with deleterious BRCA mutation (germline and/or somatic)–associated metastatic mCRPC who have been treated with androgen receptor (AR)–directed therapy and a taxane-based chemotherapy.

PARP inhibitors have been a welcome additional treatment option available for eligible mCRPC patients, and I'm pleased that this publication provides additional detail about the potential clinical benefit of Rubraca for patients…These additional data presented in this publication provide physicians important information to inform treatment decisions for their eligible patients."

The open-label, multicenter, international TRITON2 study, enrolled male patients with mCRPC associated with 1 of 13 homologous recombination repair gene alterations. Patients had disease progression on androgen receptor (AR)–directed therapy and 1 prior taxane-based chemotherapy.

Overall, the efficacy and safety populations for TRITON2 included 115 BRCA-positive patients with or without measurable disease. The median patient age was 72 (range, 50-88), 74% of

[1] See Beltram, HMS April 2021. Precision Medicine in Prostate Cancer

[2] https://www.urologytimes.com/view/pivotal-rucaparib-mcrpc-data-published

[3] https://www.researchgate.net/publication/313900832_PARP_and_Prostate_Cancer

patients were white, and all except 2 patients had an ECOG performance status <2. The median baseline PSA was 61.1 ng/mL and 67% of patients had a Gleason score ≥8 at baseline. Across the population, 62 patients had measurable disease at baseline and 53 patients had nonmeasurable disease.

1.2.2 PROfound Trial

The PROfound Trial targeted a larger group as follows[4]:

BRCA1, BRCA2, ATM, BARD1, BRIP1, CDK12, CHEK1/2, FANCL, PALB2, PPP2R2A, RAD51B/C/D, RAD54L

As has been noted, the PROfound trial consisted of the following[5]:

I want to quickly review the clinical trial design of PROfound, because sometimes it can be a bit confusing, but frankly, it doesn't need to be that way. It's fair to say that this was an mCRPC [metastatic castration-resistant prostate cancer] population. They had to have had a qualifying HRR mutation of 15 genes that were prespecified. That was largely because of great work that had been done in earlier trials known as TOPARP-A and TOPARP-B. We divided patients if they had 1 of these 15 gene alterations and had mCRPC, progressing on a novel hormonal agent. They could have also had a prior taxane.

Cohort A were those with mutations in BRCA1, BRCA2, or ATM. There were 245 patients.

Cohort B included the other 12 gene alterations in the panel.

The patients were randomized 2:1 in either cohort A or cohort B. They would receive 300 mg of olaparib, which is 2 tablets twice a day, vs a control. In the control group, if they had had abiraterone, they'd get enzalutamide. If they had previously been on enzalutamide, they'd get abiraterone. Similarly, in cohort B, with the other 12 alterations, the same randomization was used. The primary end point was rPFS. If indeed this was met, we would look at the objective response rates from a hierarchical standpoint analysis, which was done with a blinded independent central review. Subsequently, rPFS was analyzed for cohorts A and B. We then looked at other aspects, such as time to pain progression. Very interestingly, we also looked at overall survival in cohort A.

Also see deBono et al for detailed results.

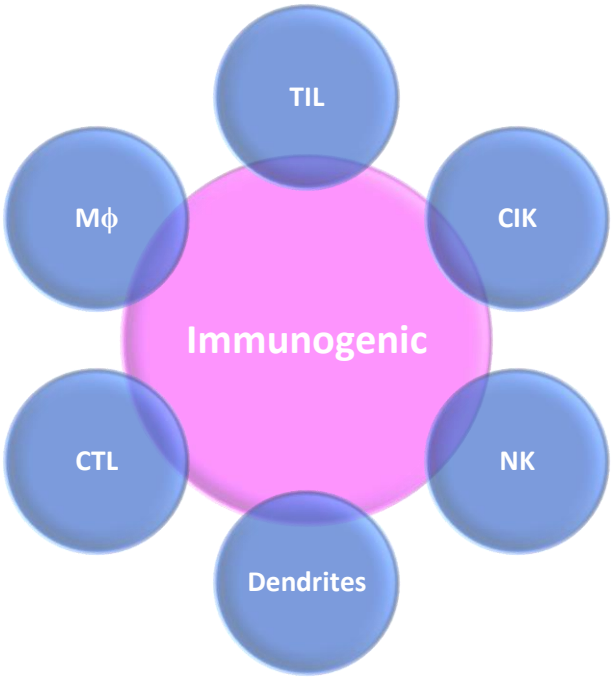
1.3 IMMUNOGENIC

Immunotherapy has become a dominant focus in many current cancers. It ranges from monoclonal antibodies, Mabs, focusing on specific targets to a Mab blocking certain ligands

[4] https://clinicaltrials.gov/ct2/show/NCT02987543?term=NCT02987543&draw=2&rank=1

[5] https://www.urologytimes.com/view/a-look-at-the-profound-trial

such as PL-1 and CTLA5. We demonstrate below some examples of the cells of the innate and adaptive system which are used:



Various epitopes have been examined and are also available[6].

1.4 Overview

The intent of this report is to use PSMA as a paradigm for other unique surface markers that can be used in various therapeutic applications. PSMA is just a single example. There are multiple such examples. The report proceeds as follows:

1. We examine PSMA and its functions. It is a surface protein and thus can be targeted. Moreover, it appears on all PCa cells especially those which have been metastasized. Thus, PSMA may be a generalized target.

2. We then examine PSMA downstream inner paths and focus on AKT and its impact. This is a complex set of issue since other genes and their products play a highly interactive role.

3. We then examine immunotherapeutic approaches. First are antibodies since we can target PSMA. This include polyspecific Abs. We believe that a great deal of specificity can be obtained by the multiple targeting or PSMA and other surface proteins. Second, we examine CAR-T cells as an example of exciting the immune system to aggressively attack the PSMA cells.

[6] https://www.iedb.org/

4. We then consider CRISPRs. This examination is related to gene modification of the targeted cells. Perhaps, for example, we could deliver CRISPRs to malignant cells for gene self-destruction. This is akin to what is done in prokaryotes.

2 PSMA

PSMA is also known as the Prostate Specific Membrane Antigen, is a surface protein which is highly expressed on prostate cells, especially those that are androgen resistant and metastatic. It has been used as a target for PET scans and somewhat for prognostic evaluation. The effects of PSMA have been understood to some degree whereas its expression control does not yet seem to be fully understood. PSMA is both correlative and causative of PCa metastatic growth. It also presents an interesting cell target for a variety of therapeutic strategies.

2.1 GENE AND PROTEIN

We begin with the NCBI definition which notes[7]:

This gene encodes a type II transmembrane glycoprotein belonging to the M28 peptidase family. The protein acts as a glutamate carboxypeptidase on different alternative substrates, including the nutrient folate and the neuropeptide N-acetyl-l-aspartyl-l-glutamate and is expressed in a number of tissues such as prostate, central and peripheral nervous system and kidney.

A mutation in this gene may be associated with impaired intestinal absorption of dietary folates, resulting in low blood folate levels and consequent hyperhomocysteinemia. Expression of this protein in the brain may be involved in a number of pathological conditions associated with glutamate excitotoxicity.

In the prostate the protein is up-regulated in cancerous cells and is used as an effective diagnostic and prognostic indicator of prostate cancer. This gene likely arose from a duplication event of a nearby chromosomal region. Alternative splicing gives rise to multiple transcript variants encoding several different isoforms.

We now present a summary of what is understood about PSMA. As Caromile et al note:

*PSMA is a **750–amino acid type II transmembrane peptidase enzyme** that is encoded by the folate hydrolase 1 (FOLH1) gene. Although PSMA is also known as glutamate carboxypeptidase II, N-acetyl-Laspartyl-L-glutamate peptidase I, and N-acetylaspartylglutamate peptidase, those studying PCa or general oncology commonly use the term PSMA, which will be used here.*

It has been shown that PSMA is present in low amounts on prostate epithelial cells and is progressively up-regulated during disease progression in prostate tumors, in which it correlates negatively with prognosis and consequently may be a promising tool for the diagnosis, detection, localization, and treatment of PCa.

Currently, PSMA is used as an immunoscintigraphic target in the clinic to direct therapy to androgen-independent prostate tumors. RNA aptamers selectively targeting PSMA enzymatic activity have also been successful in slowing primary tumor growth in murine models.

[7] https://www.ncbi.nlm.nih.gov/gene/2346

Although we have previously shown that endothelial-expressed PSMA regulates angiogenesis and retinal neovascularization primarily via b1 integrin–mediated cell adhesion, an important functional role for PSMA in PCa has not been demonstrated.

Caromile et al continue:

Here, we report that expression of PSMA in prostatic epithelial cells directly underlies prostate tumor progression in vivo. We found that tumors in wild-type animals were larger and of higher grade with a higher microvessel density as compared to tumors in the PSMA knockout animals, which is consistent with our previous results implicating PSMA as an angiogenic regulator.

In addition, PSMA-positive tumor cells were viable at greater distances from the vasculature than their PSMA knockout counterparts, suggesting that cell-intrinsic survival components also contribute to tumor growth.

Accordingly, wild-type tumors expressed relatively greater amounts of IGF-1R and exhibited greater activation of the phosphatidylinositol 3-kinase (PI3K)–AKT pathway, whereas tumors lacking PSMA not only had decreased IGF-1R expression but also had diverted signaling downstream of PI3K-AKT to the mitogen-activated protein kinase (MAPK)–extracellular signal–regulated kinases 1 and 2 (ERK1/2) pathway, consistent with a PSMA-dependent signaling switch.

Moreover, manipulation of PSMA expression in mouse TRAMP-C1 cell lines and human PCa cell lines recapitulated this change in signaling. Analysis of publicly available gene expression data sets from PCa samples confirmed that high PSMA expression was predictive of a high Gleason score.

In addition, patient samples with high PSMA expression and high Gleason scores displayed a prosurvival gene expression signature with increased expression of the antiapoptotic marker survivin and IGF-1R, consistent with a role for PSMA in the regulation of signal transduction in human PCa disease as well. Therefore, in addition to its role as a PCa marker and target, our results indicate that increasing amounts of PSMA in prostate tumor epithelium serve to drive prosurvival mechanisms and thus identify it as a functional regulator of prostate tumor progression. These findings also suggest that PSMA-positive tumors may be more sensitive to PI3K pathway inhibitors and less sensitive to MAPK pathway inhibitors.

2.2 FUNCTIONS

What function does PSMA play? Science Signalling notes Conway et al who observe:

Prostate-specific membrane antigen (PSMA) is so-named because its expression is enhanced in advanced prostate carcinomas, where its increased presence correlates with a poor prognosis. The protein is also called glutamate carboxypeptidase II and is a transmembrane protein with peptidase activity.

PSMA has been found in endothelial cells in tumor vasculature. Given roles of other peptidases in angiogenesis*, Conway et al. explored the possibility of such a role for PSMA. They used an in vivo angiogenesis assay in knockout mice lacking PSMA to show that loss of the PSMA protein inhibited formation of new blood vessels.*

Proteolysis contributes to remodeling of the extracellular matrix that is necessary for angiogenesis, but further studies by the authors suggest that PSMA may instead be part of a complex regulatory loop that controls integrin signaling and activation of the p21-activated kinase 1 (PAK1). In vitro cell invasion studies with PSMA-null cells or with inhibitors of the enzyme showed that PSMA has an important role in cell invasion and in signaling from β1 integrins to focal adhesion kinase (FAK) and PAK1.

The authors confirmed that PSMA interacts with the actin-binding protein filamin A. Disruption of this interaction with a peptide designed to compete with PMSA for binding to filamin A decreased the peptidase activity of PMSA and decreased phosphorylation of PAK1 in cultured cells. PAK1 also interacts with filamin A, and the authors propose that it may compete with PMSA for binding to filamin A.

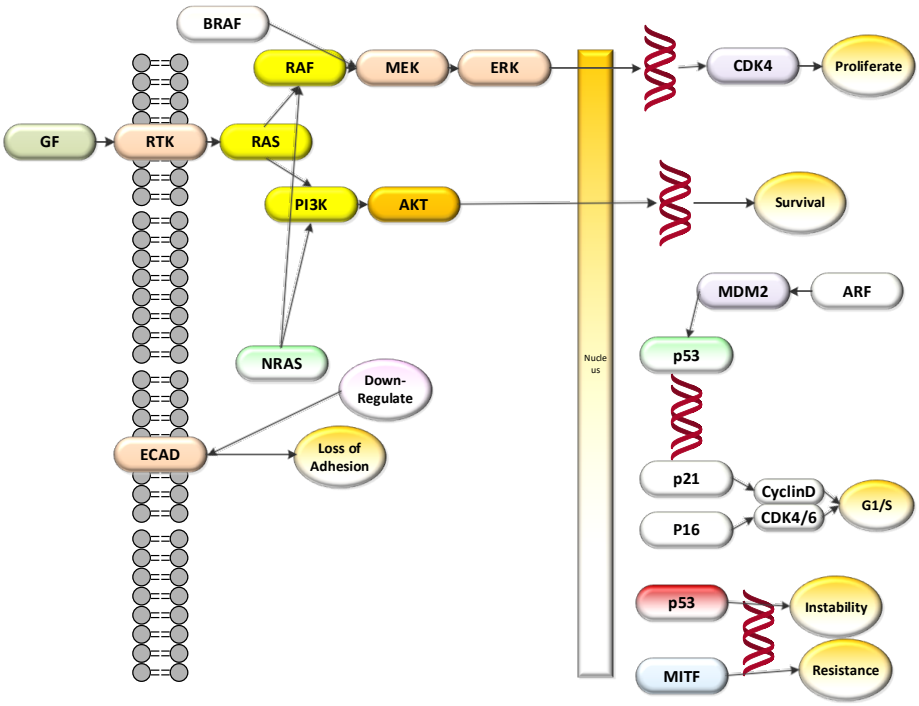
The interaction of PMSA and the cytoskeletal protein filamin A may allow a feedback signal from integrin β1 and PAK to keep PMSA activity in check. Inhibition of PAK by expression of a peptide corresponding to its autoinhibitory domain enhanced association of PMSA with filamin A, increasing its peptidase activity. Further understanding of PMSA's roles in control of angiogenesis may allow new strategies to inhibit angiogenesis in cancers and other diseases to which it contributes.

Thus PSMA can become a significant driver of a multiplicity of downstream proteins and genes.

2.3 DOWNSTREAM PATHWAYS

PSMA is also known as the Prostate Specific Membrane Antigen. It is a putative target for attacking malignant prostate cancer cells. There has been recent interest in this transmembrane protein as a target for various imaging modalities. Moreover, it has an interest as a target for a multiplicity of therapeutic modalities. We examine this marker as a means for several of these therapeutic modalities. The objective is to consider how we can “engineer” a therapeutic strategy using the many tools now available.

One of the targets for PSMA is AKT. We show below a generic flow on actions with AKT at a central role.



As Kaittanis et al have noted:

Prostate-specific membrane antigen (PSMA) or folate hydrolase 1 (FoLH1) is highly expressed on prostate cancer. Its expression correlates inversely with survival and increases with tumor grade. However, the biological role of PSMA has not been explored, and its role in prostate cancer remained elusive. Filling this gap, we demonstrate that in prostate cancer,

PSMA initiates signaling upstream of PI3K through G protein–coupled receptors, specifically via the metabotropic glutamate receptor (mGlur).

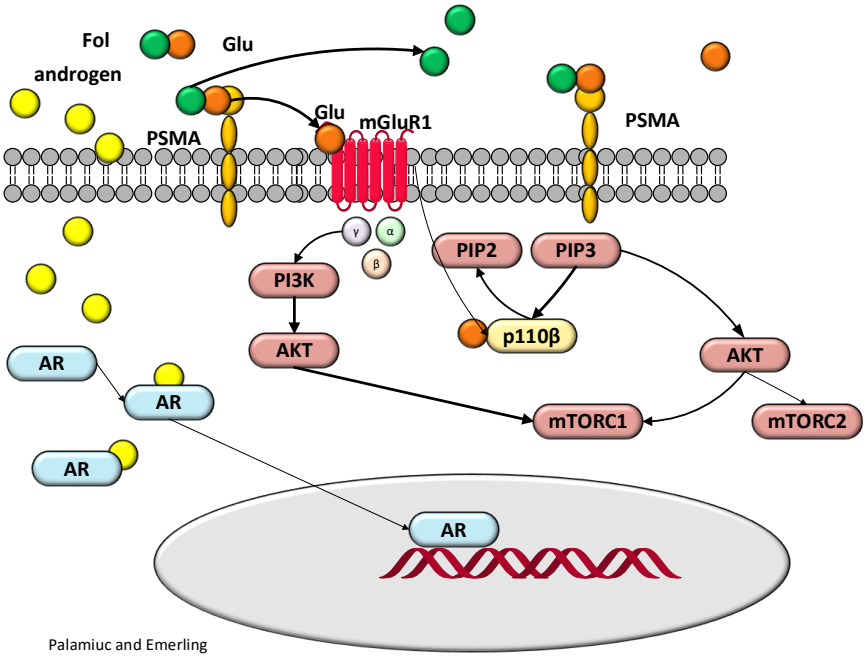
PSMA's carboxypeptidase activity releases glutamate from vitamin B9 and other glutamated substrates, which activate mGlur I. Activated mGlur I subsequently induces activation of phosphoinositide 3-kinase (PI3K) through phosphorylation of p110β independent of PtEn loss. the p110β isoform of PI3K plays a particularly important role in the pathogenesis of prostate cancer, but the origin of its activation was so far unknown.

PSMA expression correlated with PI3K–Akt signaling in cells, animal models, and patients. We interrogated the activity of the PSMA–PI3K axis through positron emission tomography and magnetic resonance imaging. Inhibition of PSMA in preclinical models inhibited PI3K signaling and promoted tumor regression. our data present a novel oncogenic signaling role of PSMA that can be exploited for therapy *and interrogated with imaging*

What is attractive in this case is that there appears in PSMA to be a cell surface marker targetable in malignant cells. We examine this marker in some detail and then examine ways in which it can be utilized in a therapeutic manner. For example, we can use PSMA as an epitope for immunotherapeutic attack. We could also use it as a target for viral insertion. Thirdly we could

use bi-specific antibodies for the delivery of cancer attacking therapeutics, in short it becomes a useful target for a variety of therapeutic application.

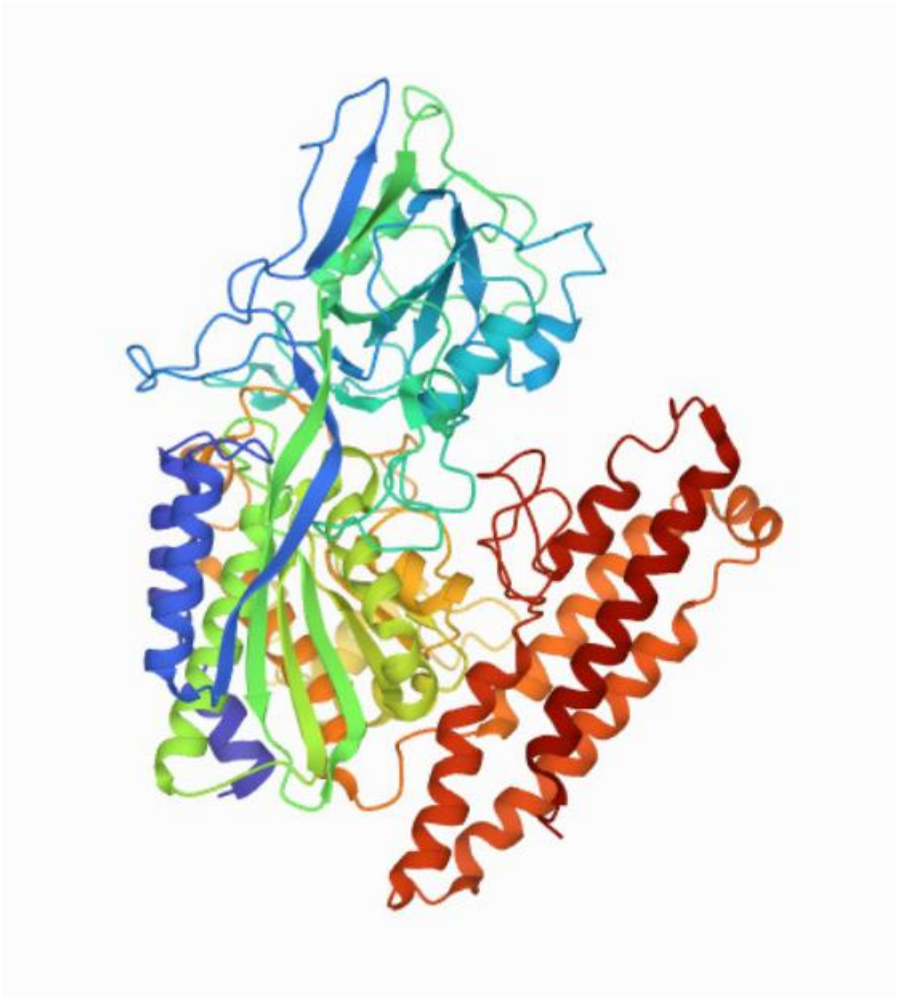
The authors, Palamiuc and Emrling, also note the dynamics using the PSMA work of Kaittanis et al, as follows:



Note the PSMA releases glutamate from the bound form and this glutamate then binds to the receptor and activates Akt. We shall examine this in detail in the next section. Now PSMA protein[8] containing 695 nucleic acids is shown below.

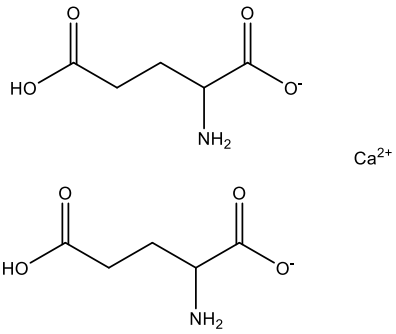
Details of the PSMA protein structure are shown in the following.

[8] https://www.rcsb.org/structure/1Z8L

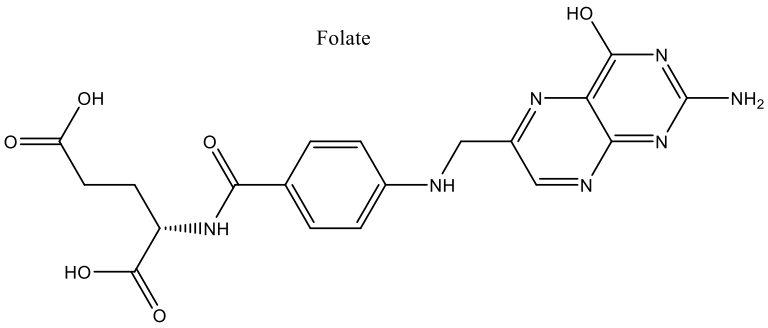


We also provide below the chemical forms of Glu and Fol as are effective in this model.

Glutamate



Folate



3 PSMA, AKT AND PI3K

We now examine the impact of PSMA activation in turn of Akt. Akt can be a potent player in many cancers especially prostate. PSMA as we shall see is a strong activator of Akt and its downward paths. PSMA is the product of the FOLH1 gene, the folate hydrolase. Recall as NCBI notes[9]:

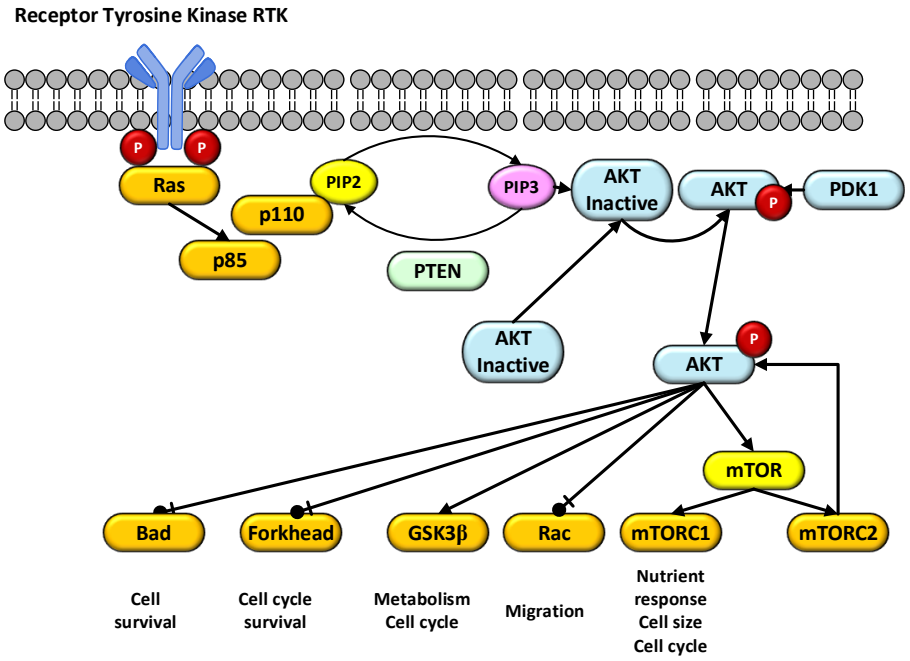
This gene encodes a type II transmembrane glycoprotein belonging to the M28 peptidase family. The protein acts as a glutamate carboxypeptidase on different alternative substrates, including the nutrient folate and the neuropeptide N-acetyl-l-aspartyl-l-glutamate and is expressed in a number of tissues such as prostate, central and peripheral nervous system and kidney. A mutation in this gene may be associated with impaired intestinal absorption of dietary folates, resulting in low blood folate levels and consequent hyperhomocysteinemia. Expression of this protein in the brain may be involved in a number of pathological conditions associated with glutamate excitotoxicity.

In the prostate the protein is up-regulated in cancerous cells and is used as an effective diagnostic and prognostic indicator of prostate cancer.

This gene likely arose from a duplication event of a nearby chromosomal region. Alternative splicing gives rise to multiple transcript variants encoding several different isoforms.

As we observed before, PSMA is a key player in the activation of the Akt pathway. In a paper by Carnero the author demonstrates the steps activated by Akt that lead to the dominant features in cancer. The figure is depicted below:

[9] https://www.ncbi.nlm.nih.gov/gene/2346



See Carnero

Carnero terms Akt the "Master" kinase. The author further notes:

AKT enhances cell survival by blocking the function of proapoptotic proteins. AKT negatively regulates the function or expression of several proapoptotic proteins that inactivate prosurvival Bcl-2 family members.

Survival factors stimulate AKTmediated phosphorylation of BAD, and this creates a binding site for 14-3-3 proteins, which sequester BAD from its target proteins.

AKT also inhibits the expression of BIM through effects on transcription factors such as FOXO and p53. AKT phosphorylates the FOXO family members FOXO, FOXO3a and FOXO4 [60] while they are nuclear, creating a binding site for 14- 3-3 proteins, which then triggers their export from the nucleus. By this mechanism, AKT blocks FOXO-mediated transcription of target genes that promote apoptosis, cell-cycle arrest, and metabolic processes. AKT also promotes survival by phosphorylating MDM2, an E3 ubiquitin ligase that accelerates p53 degradation. AKT phosphorylates MDM2, promoting translocation of MDM2 to the nucleus where it negatively regulates p53 function

Two transcriptional targets of p53, Puma and Noxa proteins, appear to be essential in p53-induced apoptosis. Likewise, AKT has also been found to phosphorylate S196 on human procaspase-9, which is correlated with a decrease in the protease activity of caspase-9 in vitro. Additionally, AKT exerts some of its cell-survival effects through the modification of nutrient uptake and metabolism.

One of the best-conserved functions of AKT is its role in cell mass increase through activation of the mTOR complex 1 (mTORC1 or the mTOR-raptor complex), which is regulated by both

nutrients and growth factor signaling. mTORC1 is a critical regulator of translation initiation and ribosome biogenesis and plays an evolutionarily conserved role in cell growth control [67]. The enhanced sensitivity of cancer cells with hyperactive PI3K/AKT to mTORC1 inhibitors illustrates the importance of mTORC1 activation downstream of AKT.

AKT activates mTORC1 indirectly by inhibiting TSC2, thereby allowing Rheb-GTP to activate mTORC1 signaling. Recently, AKT was shown to directly phosphorylate PRAS40 [69], an event important for 14-3-3 binding. PRAS40 associates with and negatively regulates mTORC1 signaling. AKT activation can stimulate proliferation through multiple downstream targets impinging on cell-cycle regulation.

Thus Akt plays a significant role in the ability of a cells to commence and continue its malignant functions.

Santoro et al have noted:

Prostate-specific membrane antigen (PSMA), best known as a prostate cancer–specific target, is a surface glycoprotein abundantly expressed on the endothelium of many solid tumors, but not on the normal vasculature. PSMA is a 750–amino acid type II membrane-bound protein transcribed from the PSMA locus, which encodes a number of splice variants, including multiple membrane-bound and cytosolic isoform. Interestingly, the ratio of membrane to cytosolic PSMA dramatically increases in prostate cancer. Recent studies have demonstrated that PSMA expression confers a proliferative advantage to tumor cells through its function as a hydrolase of poly- and gamma-glutamated folate.

As such, it is presumed that PSMA plays a metabolic role on the activated tumor endothelium. Additional functions have also been ascribed to PSMA. For example, mice lacking PSMA exhibit impaired angiogenesis as a result of defects in endothelial cell invasion. The expression of PSMA by the LNCaP prostate cancer cell line has been shown to induce the expression and secretion of IL6, which increases the proliferative potential of tumor cells. Because the tumor endothelium has been shown to be an important source of IL6, it is conceivable that PSMA signaling is also involved in the production of IL6 from these cells.

Taken together, ***these data implicate PSMA as a contributor to tumor progression, and provide strong rationale for the generation of CAR T cells against the tumor endothelial cells on which it is expressed***

This is an interesting paper regarding the use of PSMA as a target and then applying CART cells against it.

3.1 PSMA Downstream Dynamics

In a recent paper by Kaittanis et al and specifically in the review by Palamiuc and Emerling, we have:

Prostate-specific membrane antigen (PSMA) has become a popular target for developing new diagnosis tools designed to improve stratification of patients for targeted personalized therapeutic regimens (Pillai et al., 2016). PSMA is moderately expressed in several tissues, including healthy prostate tissue; however, it is greatly up-regulated in prostate cancer.

PSMA has two types of catalytic activities: NAALDase and folate hydrolase, both resulting in the release of glutamate from the enzyme substrates. Its capacity to release glutamate form N-acetyl-l-aspartyl-glutamate (NAAG) is being explored for its therapeutic potential for brain ischemic injury and several neurodegenerative disorders. investigate the folate hydrolase activity of PSMA in prostate cancer, its biological function (uncharted thus far), and, most importantly, its potential as a therapeutic target…observe a strong positive correlation between PSMA expression, disease aggressiveness, and phosphorylation of the AKT target in prostate tumor tissue from patients with localized disease.

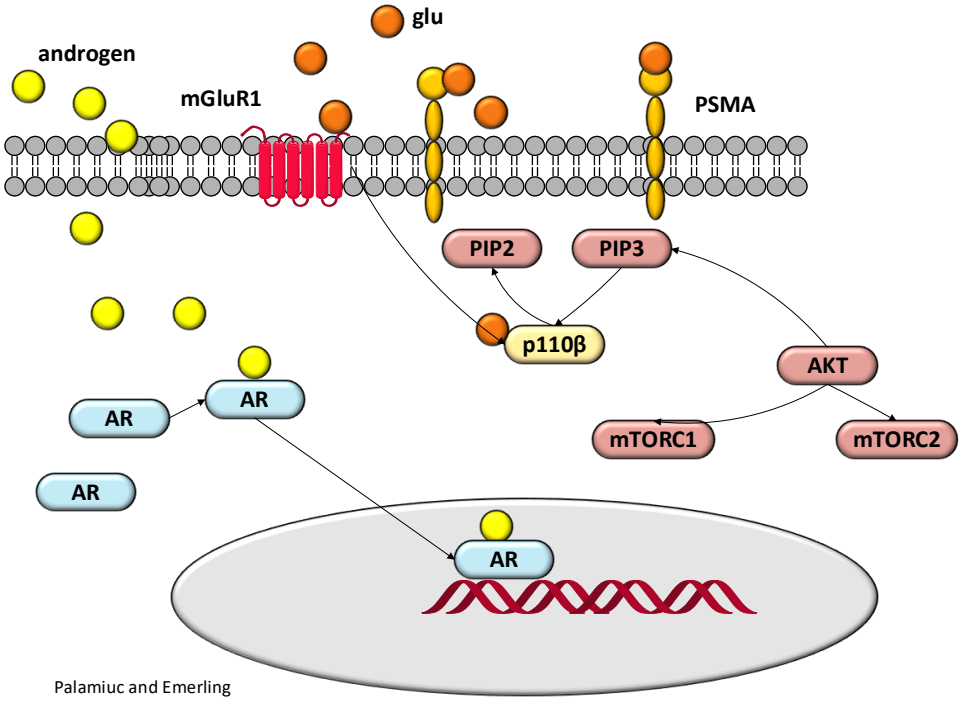
Based on this evidence, they hypothesized a significant role for PSMA in modulating signaling pathways implicated in the pathogenesis of prostate cancer, specifically the PI3K–AKT–mTOR pathway. This hypothesis is examined in detail in vitro through genetic and pharmacologic manipulation of expression and enzymatic activity of PSMA, using two different prostate cancer cell lines (LNCaP and PC3) that differ in their expression of PSMA.

Taking advantage of these systems, a series of complementary experiments demonstrated PSMA-dependent activation of AKT and subsequent increased phosphorylation of downstream targets, 4EBP1 and S6, in the absence of any known intrinsic signaling properties.

Importantly, PSMA induces AKT signaling through its enzymatic activity and subsequent glutamate release. In fact, glutamate alone is shown to activate AKT signaling. Notably, the modulatory action of PSMA on this signaling pathway is dependent on the presence of an enzymatic substrate (ea. vitamin B9), which can be abolished by 2-PMPA, a known inhibitor of PMSA.

Interestingly, …PSMA activates PI3K signaling through phosphorylation of p110β, independent of PTEN status. Furthermore, PSMA expression in samples from 76 patients showed a strong correlation with AKT, but no correlation with PTEN expression was observed

They describe the process as below which we have shown previously.



As to the above, the authors note:

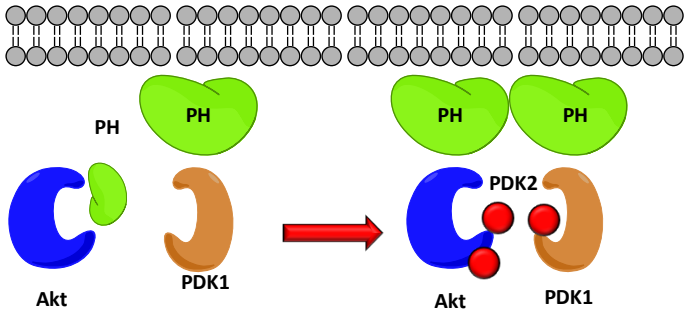
A versatile tool for prostate cancer therapy… PSMA is expressed with high specificity at the membrane of prostate cancer cells. Through its unique position and enzymatic function, it constitutes a notable target for radiolabeling.

Because of its strict correlation with AKT expression, it could prove to be the ideal tool for diagnosis and patient stratification.

Moreover, targeting PSMA inhibits PI3K signaling in prostate cancer cells; thus, combinatorial approaches with androgen pathway inhibitors and PSMA inhibitors could lead to a powerful therapeutic tool, overcoming the off-target toxic effects associated with other therapies, such as PI3K inhibitors. Combining these two applications may pave the way toward innovative PSMA-targeted theranostic approaches.

3.2 AKT AND PSMA

The binding of the initiating proteins, especially Akt, is shown via the activation below where we show the utilization of the Pleckstrin homology (PH):



Blume-Jansen

And the authors note:

Akt activation… is thought that the N-terminal PH domain[10] precludes kinase access to and phosphorylation of the activation-loop Thr308 by PDK-1. Right: PI(3)K activation results in production of PtdIns(3,4,5)P3 and PtdIns(3,4)P2, which recruits Akt to the membrane by binding to its PH domain. This exposes Thr308 for phosphorylation by PDK-1, which is already located at the membrane. An unidentified PDK-2 kinase phosphorylates Ser473 in the C terminus, which leads to full Akt activation.

Now Carver et al note:

In summary our results demonstrate that inhibition of the PI3K pathway in PTEN-negative prostate cancer results in feedback signaling to the receptor tyrosine kinase HER2/HER3 leading to activation of AR. Conversely, blockade of AR results in activation of AKT through reduced levels of FKBP5 impairing the stability of PHLPP.

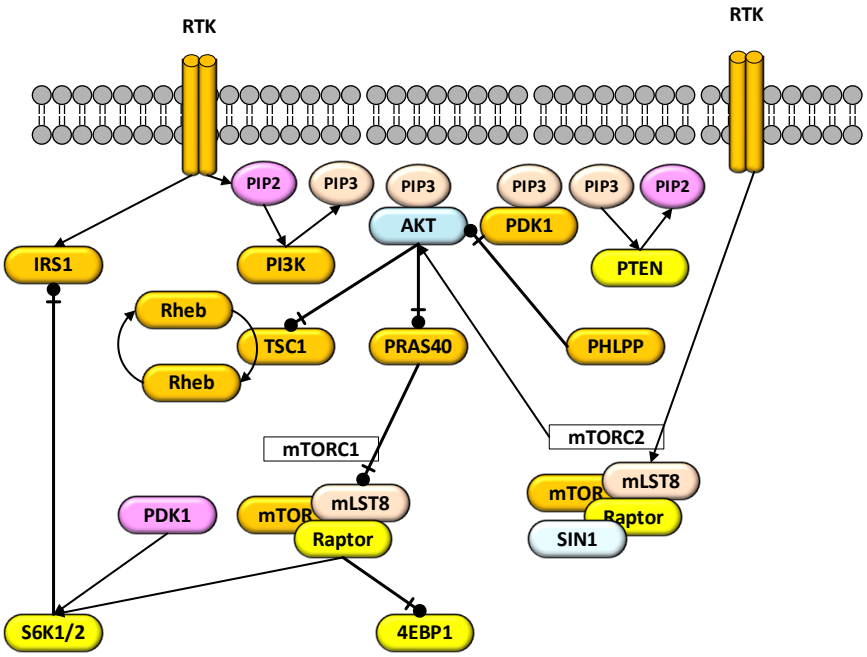
This bidirectional crosstalk between two critical survival pathways in prostate cancer provides the molecular rationale for simultaneously targeting both pathways.

The success of clinical trials evaluating PI3K pathway inhibitors in prostate cancer could be optimized by enrolling patients with documented activation of the PI3K pathway and treating in combination with appropriate AR pathway inhibition.

As a therapeutic strategy, the above observation appears to argue for the ever more popular approach of multiple therapeutic regimens.

The Manning and Cantley system for Akt is shown below (as modified):

[10] PH domain is the Pleckstrin homology domain or PHIP.



3.3 mTOR Interaction

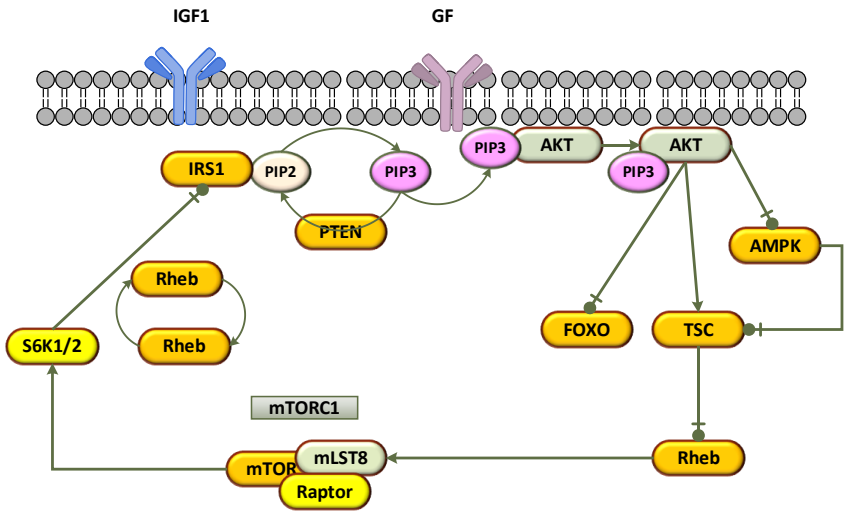
mTOR is a powerful player in many cancers[11]. The interaction with mTOR is essential to understand.

As Hay has noted:

In the PI3K/Akt/mTOR pathway, Akt is flanked by two tumor suppressors: PTEN, which antagonizes PI3K and therefore inhibits Akt, and TSC1/TSC2 heterodimer, which inhibits mTOR by inhibiting the activity of Rheb Akt activates mTOR via direct phosphorylation of TSC2 and by the inhibition of AMPK, thereby activating Rheb and mTOR-Raptor activity. Upon activation, mTOR-Raptor activates S6K and inhibits 4E-BP to accelerate mRNA translation, and also initiates feedback inhibition of Akt, which is at least in part mediated by S6K

and this is shown below:

[11] https://www.researchgate.net/publication/338412510_mTOR_Target_of_Opportunity



See Hay, 2005

From Hay we have another view of these pathways emphasizing the mTOR importance[12] and Hay also notes:

The downstream effector of PI3K, Akt, is frequently hyperactivated in human cancers. A critical downstream effector of Akt, which contributes to tumorigenesis, is mTOR. In the PI3K/Akt/mTOR pathway, ***Akt is flanked by two tumor suppressors: PTEN, acting as a brake upstream of Akt, and TSC1/TSC2 heterodimer, acting as a brake downstream of Akt and upstream of mTOR.***

In the absence of the TSC1/TSC2 brake, mTOR activity is unleashed to inhibit Akt via an inhibitory feedback mechanism. Two recent studies used mouse genetics to assess the roles of PTEN and TSC2 in cancer, underscoring the importance of AktmTOR interplay for cancer progression and therapy…

The implication of Akt-mTOR interactions for cancer therapy The studies … underscore the importance of Akt-mTOR interrelationships for the progression and therapy of cancer. Akt and mTOR are linked to each other via positive and negative regulatory circuits, which restrain their simultaneous hyperactivation. This might have been evolved as a protection mechanism to inhibit uncontrolled cell survival and proliferation. As indicated above, the feedback inhibition of Akt induced by hyperactivation of mTOR rapamycinsensitive activity was attributed to the inhibitory effect of S6 kinase on IRS-1 downstream of IGF-1 and insulin receptors. However, the inability of serum or PDGF to overcome this inhibition, together with the … that the feedback inhibition also occurs in vivo in mouse tissues that are exposed to a variety of growth factors, which activate their cognate receptors and PI3K, points to a more general mechanism.

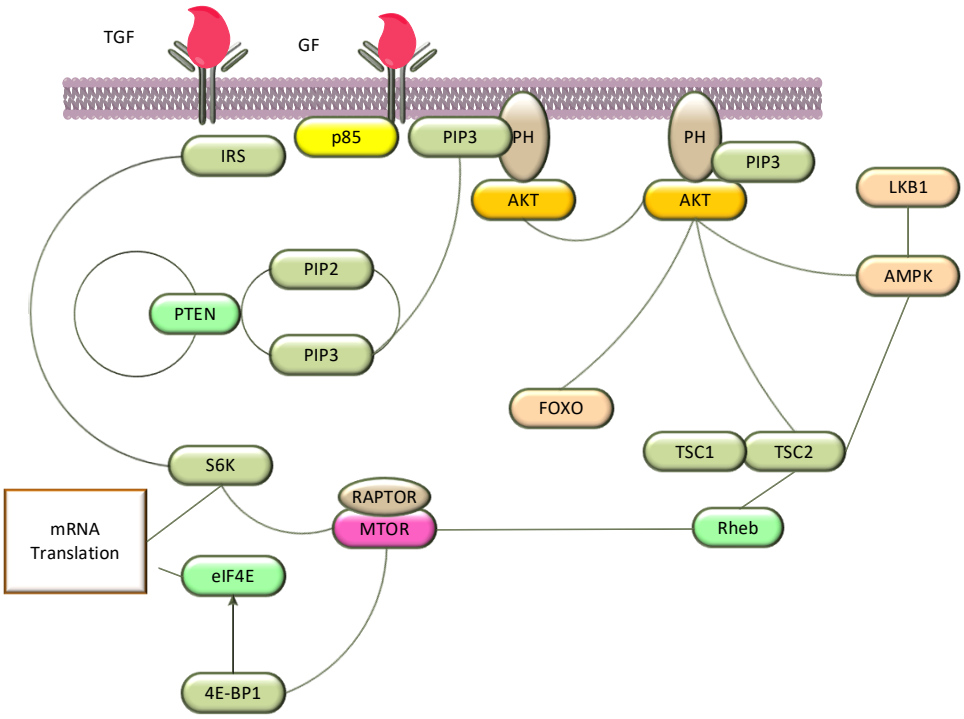
Since haplodeficiency of PTEN is sufficient to alleviate the feedback inhibition, one possibility is that hyperactivation of the rapamycin-sensitive activity mTOR could elevate PTEN activity.

[12] https://www.researchgate.net/publication/338412510_mTOR_Target_of_Opportunity

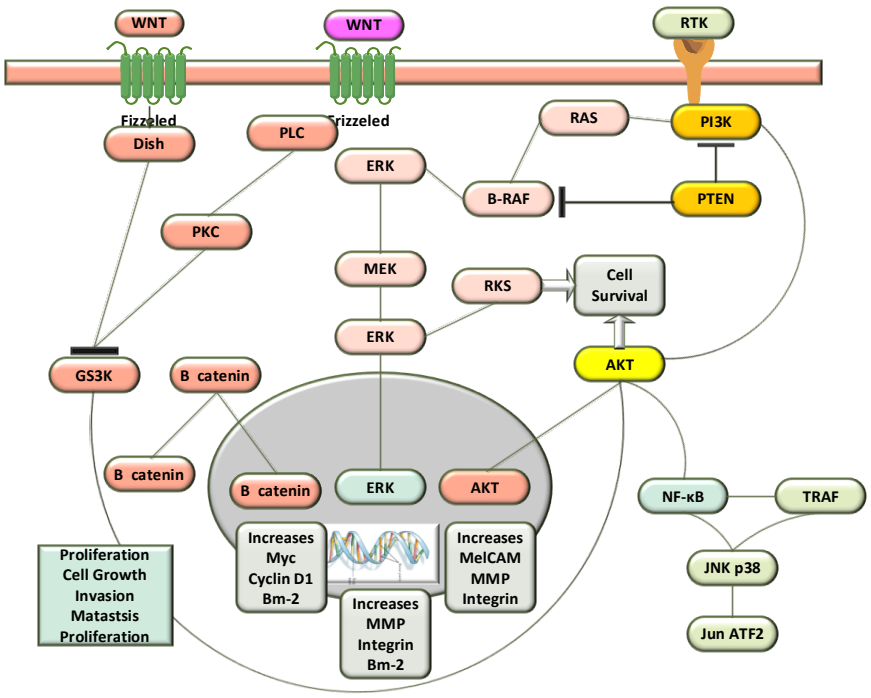
Alternatively, the existence of two mTOR complexes may explain the inhibitory feedback. As indicated above, mTOR exists in two separate complexes, the mTOR-Raptor, a rapamycin-sensitive complex, which is activated by Akt, and the mTOR-Rictor, a rapamycin-insensitive complex, which is activated by growth factors and possesses PDK2 activity. If mTOR-Rictor is indeed the principal PDK2, following growth factor stimulation, the mTOR-Rictor complex activates Akt.

When activated, AKT inhibits the activity of TSC1/TSC2 heterodimer to activate Rheb, which in turn promotes the formation of mTOR-Raptor complex to activate the rapamycin-sensitive activity of mTOR. Assuming that an equilibrium exists between these two complexes within the cell, when the mTOR-Raptor complex is formed, it could antagonize the formation of mTOR-Rictor complex and therefore reduce Akt activity

We present this formulation below:

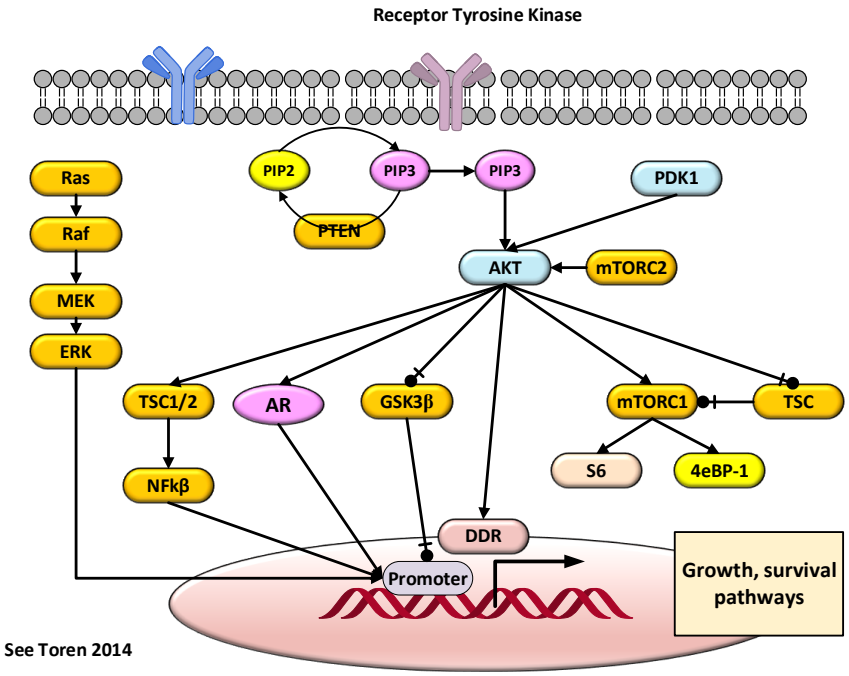


A more complete view of this process is as follows:



3.4 AR INTERACTIONS

From Torren Zoubeidi we have the following model which expressly includes AR:



As Toren and Zoubeidi note:

The PI3K/Akt pathway is an actively pursued therapeutic target in oncology. In prostate cancer, the activation of this pathway appears to be characteristic of many aggressive prostate cancers. Further, activation of the PI3K/Akt pathway is more frequently observed as prostate cancer progresses toward a resistant, metastatic disease. Signalling from this pathway activates numerous survival, growth, metabolic and metastatic functions characteristic of aggressive cancer. Biomarkers of this pathway have correlated activation of this pathway to high grade disease and higher risk of disease progression.

Therefore, there is significant interest in developing effective strategies to target this pathway in prostate cancer. In this review, we discuss the pre-clinical and clinical data relevant to targeting of the PI3K/ Akt pathway in prostate cancer. In particular, we review the rationale and relevance of co-targeting approaches against the PI3K/Akt pathway. It is anticipated that through an improved understanding of the biology of the PI3K/Akt pathway in prostate cancer, relevant biomarkers and rationale combination therapies will optimize targeting of this pathway to improve outcomes among patients with aggressive prostate cancer…

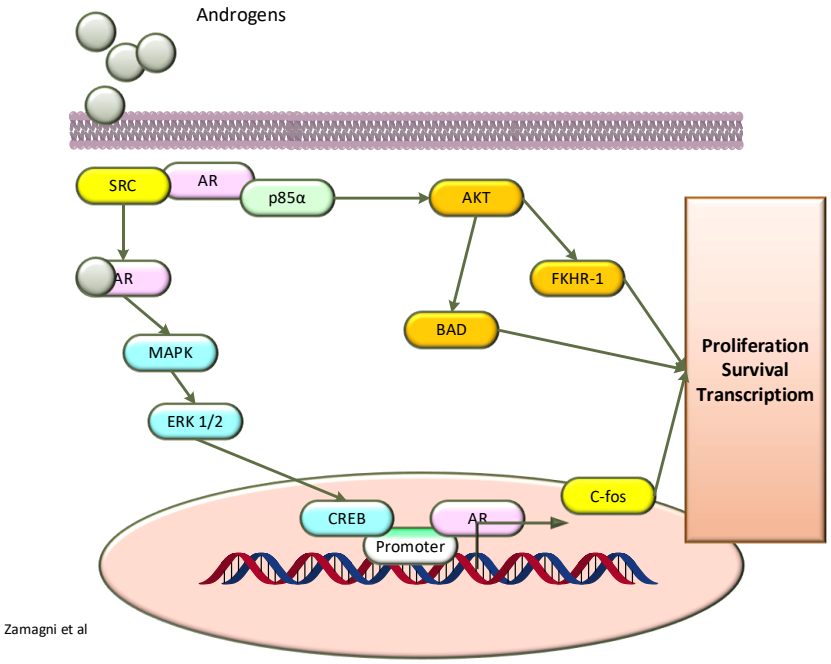
The mechanisms through which the PI3K pathway may induce carcinogenesis include the activation of growth and survival pathways.

Further, activation of this pathway may also alter epigenetic regulators, such as BIM1. The PI3K/Akt pathway has also been shown to be important to the survival and proliferation of prostate cancer stem cells.

PTEN deletion is commonly used to model prostate cancer progression in mice (24,25). PTEN loss in mice has been shown to suppress androgen-responsive genes and promote cell autonomous growth. Activation of the P3K/Akt pathway in mice may also occur using myristolated Akt or constitutive activation of p110β. In a murine subrenal xenograft model, activation of both AR and Akt has been noted to synergize to increase prostate tumour growth (27). Nonetheless, the exact role of this pathway in carcinogenesis in humans is uncertain. On the contrary, a recent genome wide sequencing analysis suggests that PTEN loss is a late-stage feature in the progression of prostate cancer. Pre-clinical studies suggest that concomitant loss of certain proteins together with PTEN loss appear to accelerate prostate cancer progression.

This has been demonstrated in mice and correlated with features of aggressiveness, such as Gleason score, in patient samples for the tumour suppressors NKX3.1, EAF2/U19, Gata3 and Sox9. B-raf and Stat3 activation and loss of SMAD4 and p53 signalling has also been shown in murine models to cooperate with PTEN loss to enhance prostate cancer progression. This complex network with other pathways highlights why monotherapy against the PI3K/Akt pathway may not be an optimal strategy.

Zamagni et al suggest the following pathway analysis:



They then note:

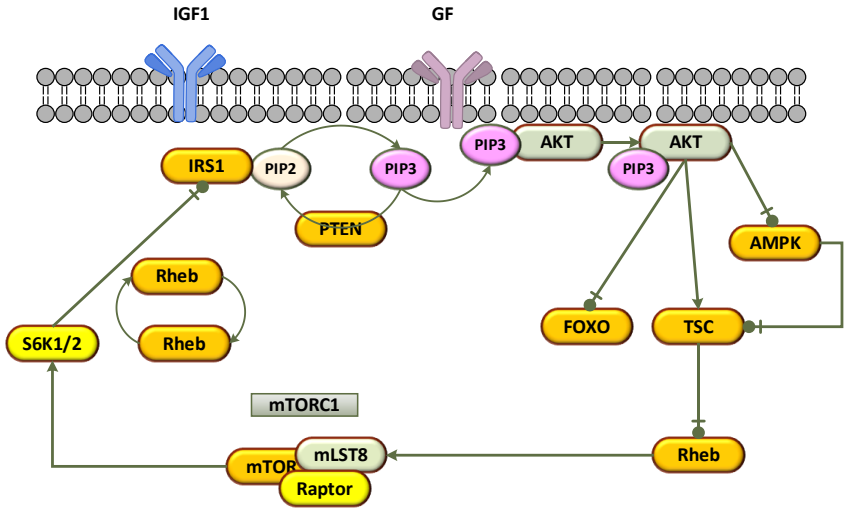
Despite the key role played by androgen receptor (AR) in tumor cell aggressiveness and prostate cancer (PCa) progression, its function in the tumor microenvironment (TME) is still controversial. Increasing studies highlight the crucial role played by TME modulation in treatment outcome and tumor cell spreading.

In this context, targeting specific constituents of the TME could be considered an alternative approach to classic treatments directed against cancer cells. Currently, androgen deprivation therapy (ADT) is a routinely adopted strategy in the management of PCa, with initial success, and consecutive fail. A possible justification to this is the fact that ADT aims to target all the transcription/translation-related activities of AR, which are typical of tumor epithelial cells. Less is still known about side effects of ADT on TME. Cancer Associated Fibroblasts (CAFs), for example, express a classic AR, mostly confined in the extra-nuclear portion of the cell.

In CAFs ***ADT exerts a plethora of non-transcriptional effects, depending by the protein partner linked to AR, leading to cell migration, proliferation, and differentiation.*** *In recent years, substantial progress in the structure-function relationships of AR, identification of its binding partners and function of protein complexes including AR have improved our knowledge of it signaling axis. Important AR non-genomic effects and lots of its cytoplasmatic binding partners have been described, pointing out a fine control of AR non-genomic pathways.*

Accordingly, new AR inhibitors have been designed and are currently under investigation. Prompt development of new approaches to target AR or block recruitment of it signaling effectors, or co-activators, is urgently needed*. The present review takes an in-depth look at current literature, furnishing an exhaustive state-of-the-art overview of the non-genomic role of AR in PCa, with particular emphasis on its involvement in TME biology*

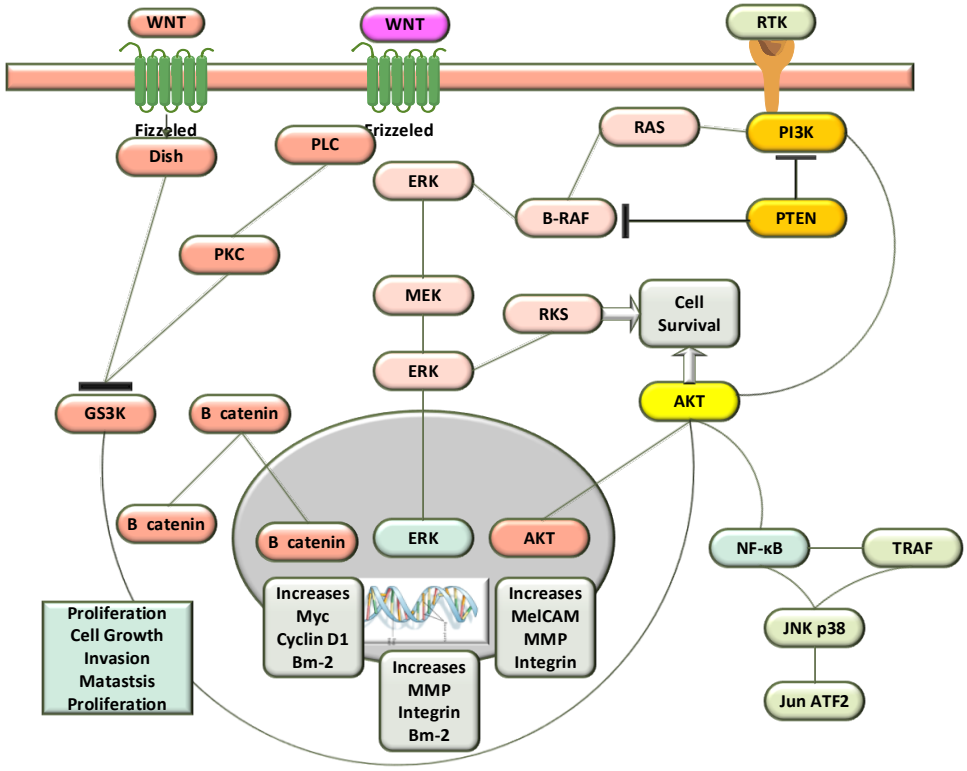
We have examined the tumor micro environment in several varying aspects[13].



See Hay, 2005

Another more complete view is shown below:

[13] See https://www.researchgate.net/publication/341788660_Fibroblasts_and_Cancer_The_Wound_That_Would_Not_Heal ,
and
https://www.researchgate.net/publication/336116071_Tumor_Associated_Immune_Cells_On_the_one_hand_and_on_the_other_hand ,



In the above we show not only the receptor tyrosine kinase but also WNT/Frizzled combination and the impact of MYC and other players resulting in cell proliferation and movement. All too often the complexity of intracellular networks can distort the putative therapeutic effects.

4 IMMUNOTHERAPY

There are a variety of immunotherapies that have been applied to dealing with PSMA as an example. We review some of them here.

4.1 ANTIBODIES

Antibodies are structured proteins that bind to an antigen and activate the immune system via T cells. Simple concept. The challenge is to find the right antigen, then get the right antibody, and then hope it does not cause overall havoc. The recent coronavirus pandemic is a simple example. Fortunately, the virus has a spike protein that attaches to the ACE2 receptor. Nicely and even elegantly engineered to then enter the cells and cause havoc. The solution is to tell the body what the antigen is, the spike and let the immune system go to work, assuming it can work. The result is an immune response via the insertion of the spike mRNA into the cell cytoplasm via an mRNA coated in PEG. Lots of knowledge and tools were available to let us do this. Could not have done so quickly a decade earlier. Now we just have to stay ahead of the bow wave, read variants.

Now the reason we were so far ahead was the use of Abs for cancer and the use of cell inserts for the same purpose. It was not the viral folks at NIH or CDC that got us there it was the independent entrepreneurial one's focusing on cancer, a much more ruthless foe. Thus, in this section we examine the use of Abs for cancer and th use of PSMA as the target antigen.

4.1.1 Bi-specifics

Simple Ab are now well known. One Ab per one Ag. But what is we can get better efficacy and specificity by targeting several Ag at once? We can now move on to bi-specific antibodies. Bi-specific antibodies have recently become more readily available and can perform multiple therapeutic effects simultaneously.

As Kaiser has noted regarding some historical elements:

Bispecific antibodies offer a third way to harness T cells. In the mid-1980s, cancer researchers began to engineer antibodies that had two tips—one matched to a cancer cell antigen and the other to a T cell surface protein called CD3. The idea was to directly link T cells to tumor cells, thereby skipping the need for T cells to learn to attack a cancer. "It's mimicking what naturally happens, but the advantage is that you can engage all T cells," not just those trained to attack the tumor, says Dirk Nagorsen, a vice president and cancer researcher at Amgen.

In 1985, the field was galvanized by two reports in Nature that such a "bispecific" antibody could destroy cancer cells in a dish; studies soon showed those antibodies could shrink tumors in mice. The drugs were hard to make. Antibodies are modular, with two identical "heavy" chains, making up the stem and half of each arm of the Y, and two identical "light" chains, each of which completes one arm. Trying to assemble bispecific antibodies from those complex components, protein chemists got 10 versions of each molecule. That outcome meant laborious

efforts to sift out the one researchers wanted the first bispecific antibody for cancer was approved in Europe in 2009. It was meant to mop up the malignant cells that cause abdominal fluid to build up in some cancer patients—but it didn't work that well, so the drug only stayed on the market a few years.

The field regained momentum, however, after Amgen snapped up Micromet in 2012 and later showed that its BiTE drug, blinatumomab (Blincyto), doubled the survival time of patients with advanced acute lymphocytic leukemia. Beginning in 2014, the Food and Drug Administration approved the drug to treat several adult and pediatric forms of the disease. Amgen is now testing BiTEs for other cancers, including myeloma and lung, prostate, and brain cancers. …

Solid tumors are a challenging target for bispecifics in part because tumors often lack a unique antigen for the antibodies to grab. Many tumors are also surrounded by blood vessels, tissue, and immune cells that form a barrier T cells can't easily penetrate.

The issue with solid tumors is critical. The most important part of a MaAb functioning is the Ag target. To be effective the target must be singular to the target and thus not one on a multiplicity of other cells. Furthermore, for solid tumor we must be able to reach the cells. This is often the most difficult part. If the drug is administered in some IV manner we then must know that the targets are adequately perfused and that there can be a ready extravasation from the blood stream to the cells. Furthermore, we need to have adequate supplies of immune cells such as CTL. Naturally we could also try to use NK cells.

But findings from mouse studies suggest some bispecific antibodies can drive T cells into tumors, says Nai-Kong Cheung of Memorial Sloan Kettering Cancer Center. His lab has systematically tweaked design factors, such as how binding sites are arranged, to learn what optimizes the molecules' potency. And some companies hope to boost the attack on solid tumors with antibodies that bind not only to CD3, but also to another receptor on T cells known as a "second signal," which stimulates the cells to grow. For years, says … has been "afraid to touch" that protein, called CD28, because of a devastating mishap: An antibody designed to bind to it made six healthy volunteers critically ill from cytokine release syndrome in a 2006 U.K. clinical trial.

Findings from new studies, however, suggest it's possible to exploit that cell growth trigger safely.

Last year in Nature Cancer, a Sanofi team reported that a "trispecific" antibody with arms matched to CD28, CD3, and a cancer antigen wiped out myeloma tumors in mice[14].

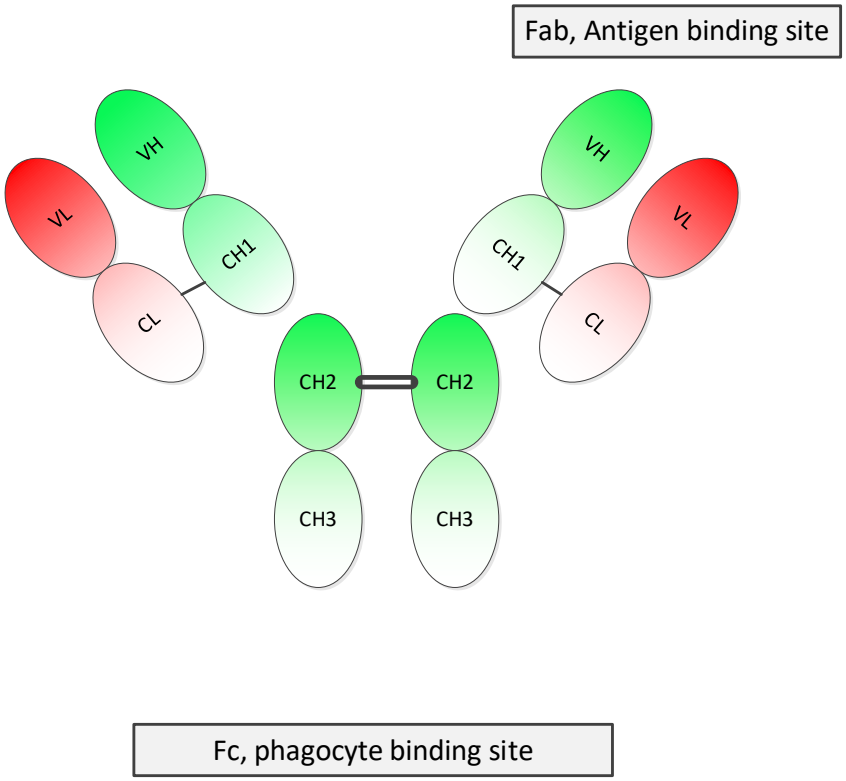
Other firms have split up the task by creating two bispecifics. One targets a tumor antigen and CD28 or another growth-signal receptor; the other binds to the tumor antigen plus CD3. "One of our hopes is that this costimulatory bispecific may help us unlock responses in solid tumors," says Lowy, whose company reported in Science Translational Medicine in January that such a two-drug combination shrank ovarian tumors and slowed prostate tumor growth in mice.

[14] See http://www.hcdm.org/index.php/molecule-information for lists of CD molecules.

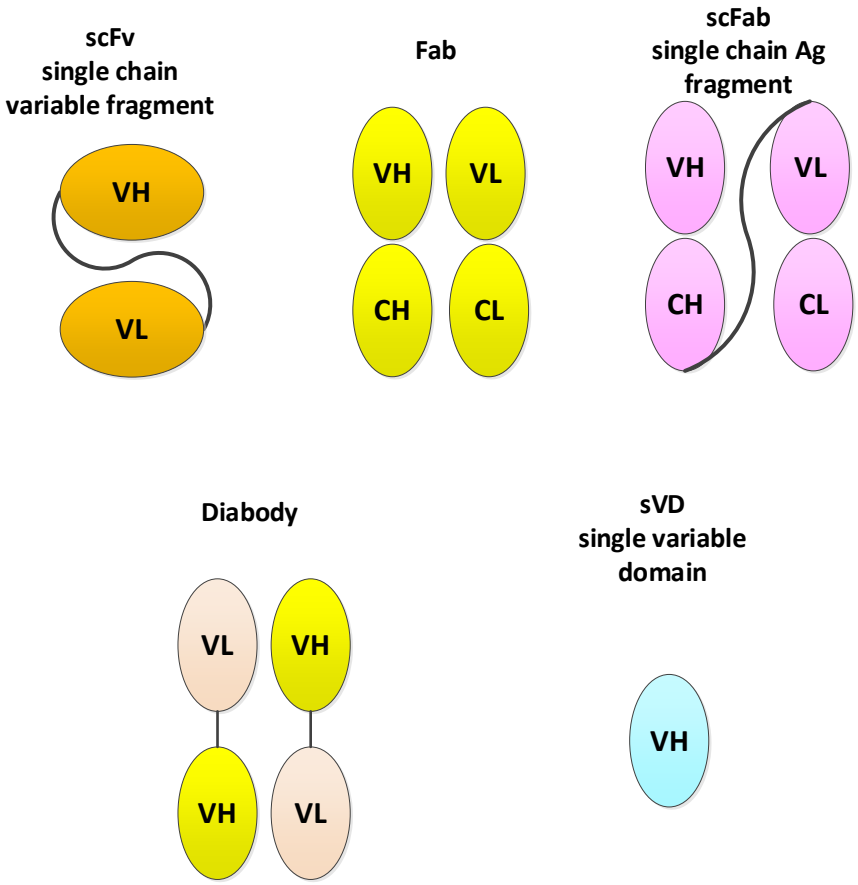
The above reference to tri-specifics is a critical observation. We shall return to this. Targeting CD28 and CD3 is but one of many targets. We shall also see that getting the correct targets will become the major challenge.

4.1.1.1 General Constructs

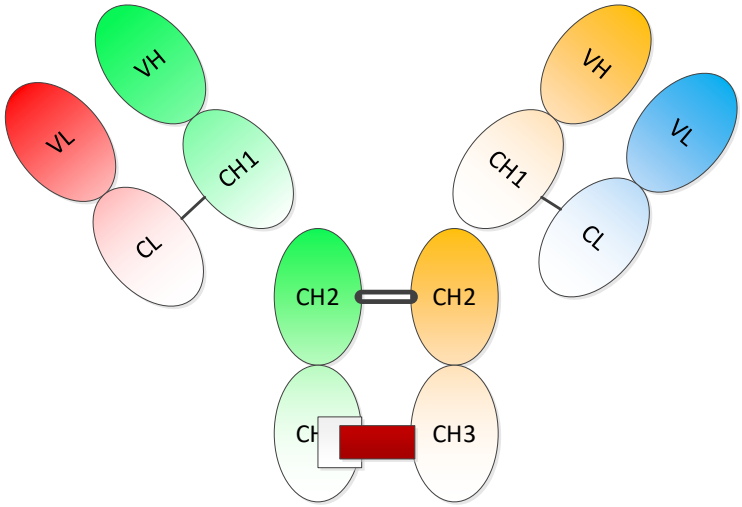
Let us start with a simple IgG antibody. It is shown below with Fab and Fc ends but also with a bond across the long chain in the middle. This Ab has a single Fc domain and thus attaching to a specific immune cells and a single Fab domain attaching to a specific Ab. The idea is that one can possibly create an Ab with multiple Ag attachments, and even ones where there is no Ac and immune attachment but all Ab attachments. Of course, one could even imagine a set of poly Ag domains and thus we would potentially have a carrier that takes some molecule such as a therapeutic and then attaches to a specific cell such as a cancer cell.



The goal is to use the above paradigm but in the context of two different Abs from two differing hybridomas. We can have various ways or motifs to assemble them and the graphic below is an example.



Thus, using these various motifs, we can assemble a wide variety of bi-specifics. In fact, these motifs can become the base set of any polyAb. Consider the modification of the classic IgG below:



Note: Knob is the red bump and hole is depression. They fit.
However 2 knobs or 2 holes will not fit

This is another variation called knobs in holes. Namely we have on the long end a solid binding protein extending outward while on the other side we have a protein inward and a matching of the proteins to lock in the structure. Furthermore, in the above case we show a variety of long and short elements creating a complex motif. bi-specifics present a large multiplicity of shapes as well as binding locations.

4.1.1.2 Various Implementations

We can now classify the variation is a variety of ways. The Table below look at IgG line, Fragment like and appended IgG or Fc. Frankly there may be many ways to classify b-specifics and we use a few different ones herein.

IgG Like Formats	Fragment Based	Appended IgG or Fc
κλ Bodies	VK/VL Format	Fv-IgG
Common LC	Single Domain	ScFv-IgG
Knob in Hole		Single Domain Ab-IgG
Charge Pair		BiTE
CH1/CL Cross Ab		DART

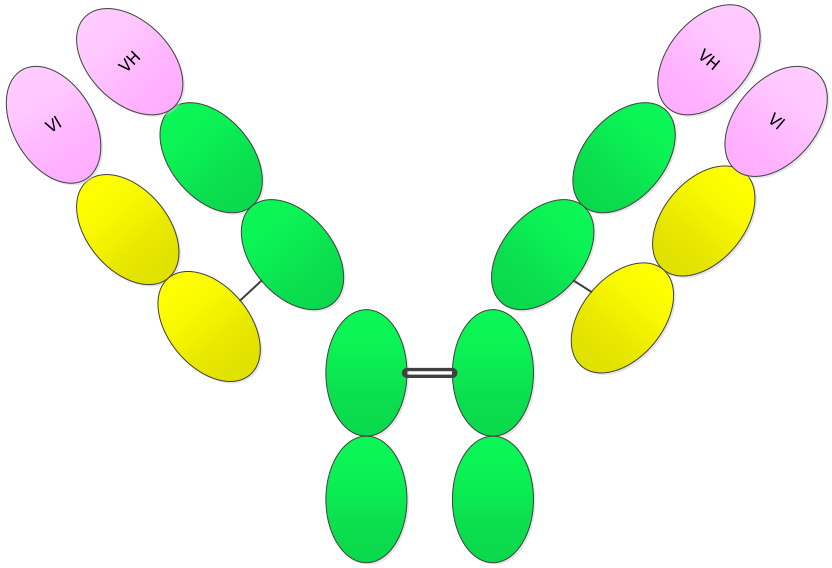
We now examine these in some shape detail.

1. Fc Based Formats

The first division is Fc based which are the direct IgG like. Namely there is an Fc domain and Fab portions. We consider the various ones here.

2. Dual Variable Domains Ig (DVD-Ig)

The DVD-Ig is shown below. This is a dual domain on both the Ab and Fc sides. The Ab sides have four variables due to the added binding domain. Note we have three on each Fab side rather than the two normally.

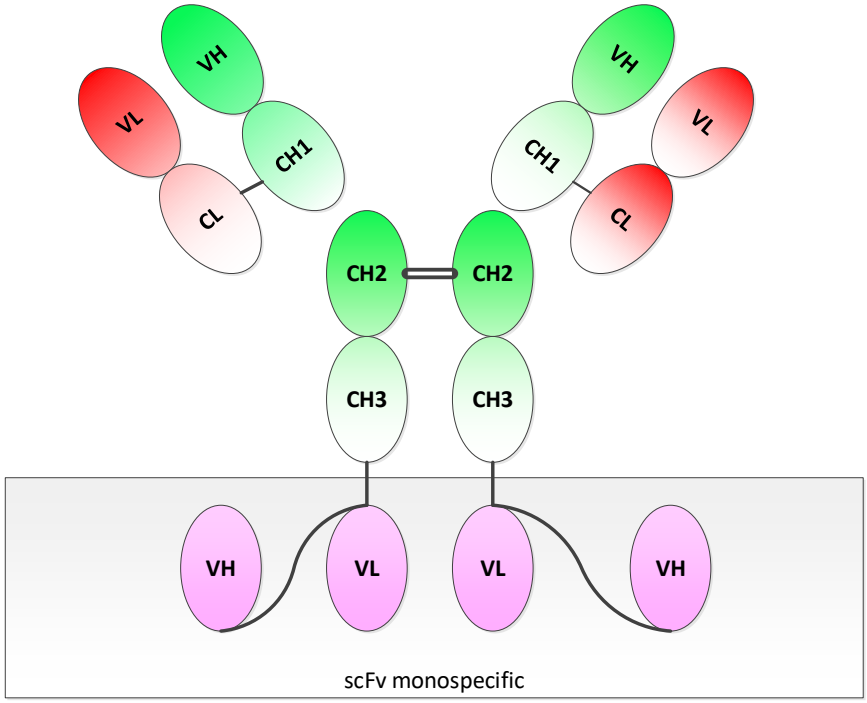


The above has been used in the case of binding VEGF and DLL4 ligands to inhibit angiogenesis in tumor cells[15].

1. scFv-Ig Fusions

This design is very complex in that it employs multiple motifs. It is symmetric but tetravalent.

[15] Note: DLL4 is found to be a gene promoting hepatocellular cancer, see Kunanopparat et al, Delta-like ligand 4 in hepatocellular carcinoma intrinsically promotes tumour growth and suppresses hepatitis B virus replication, World J Gastroenterol 2018 September 14; 24(34): 3861-3870

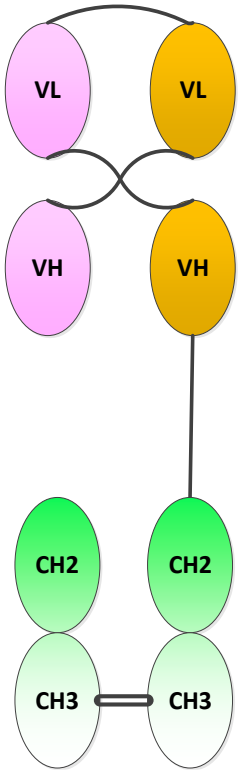


Currently this has been developed to target HER1 and cMET[16].

2. scFv-Fc Fusions

scFv-Fc fusions are a fusion process extending the use of IgG structure Ab with more complex bonding. DART is an example. DART uses a fragment of Fcs as shown below, then has then fused with a diabody atop. The diabody is two chains interlinked and with DART they are further interlinked to yield stability. It is stated that this has the greatest stability due to this interlinking.

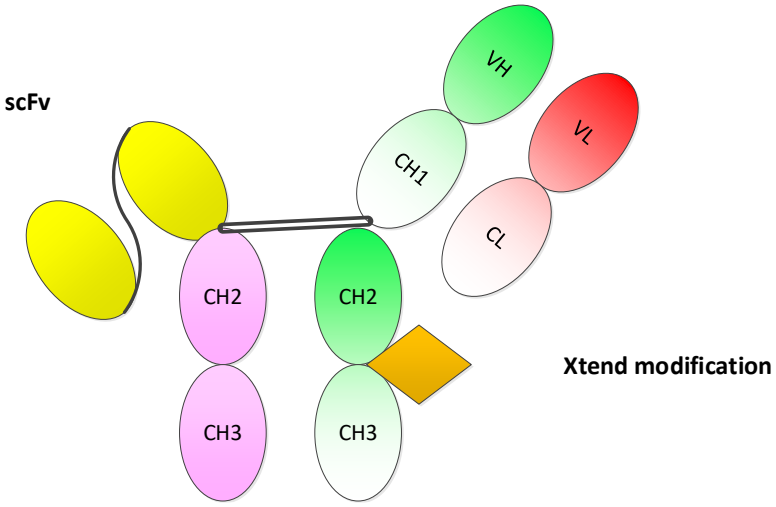
[16] HER1 is also known as EGFR. The protein encoded by this gene is a transmembrane glycoprotein that is a member of the protein kinase superfamily. This protein is a receptor for members of the epidermal growth factor family. EGFR is a cell surface protein that binds to epidermal growth factor, thus inducing receptor dimerization and tyrosine autophosphorylation leading to cell proliferation. Mutations in this gene are associated with lung cancer. (see https://www.ncbi.nlm.nih.gov/gene/1956) cMET, also MET, encodes a member of the receptor tyrosine kinase family of proteins and the product of the proto-oncogene MET. The encoded preproprotein is proteolytically processed to generate alpha and beta subunits that are linked via disulfide bonds to form the mature receptor. Further processing of the beta subunit results in the formation of the M10 peptide, which has been shown to reduce lung fibrosis. Binding of its ligand, hepatocyte growth factor, induces dimerization and activation of the receptor, which plays a role in cellular survival, embryogenesis, and cellular migration and invasion. Mutations in this gene are associated with papillary renal cell carcinoma, hepatocellular carcinoma, and various head and neck cancers. (see https://www.ncbi.nlm.nih.gov/gene/4233)



DART has the T cell targeting capacity due to the retaining of the Fc region and the variable ends allow for complex multi receptor binding. In effect this is a T cell guide Ab.

1. XmAb

XmAb has an Fc domoain but there is an attached amino acid complex which alleges extends the lifetime of the Ab. The variable end is bi-specific with an scFv element and a standard format.



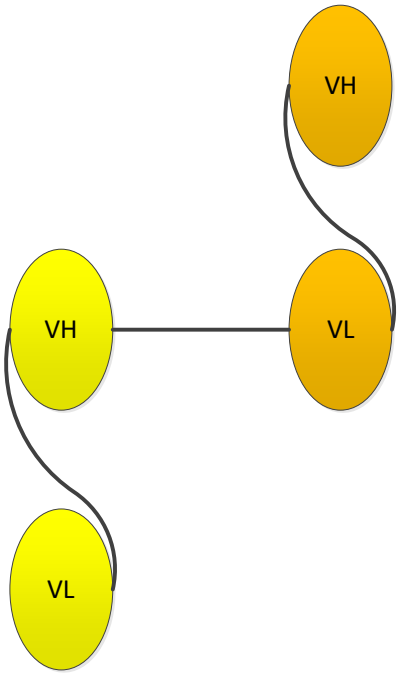
This has a Fab target of LAG-3 and a scFv target of CTLA-4[17].

3. Fragment Based, Fab

The second class is a non-Fc based class of Fab variants.

1. BiTE

BiTE is a more mature bispecific. It contains the two motifs that we see below and no Fc element.



The Bispecific T cell approach has seen limited use. As Huehls et al note:

Bispecific T cell engagers are a new class of immunotherapeutic molecules intended for the treatment of cancer. These molecules, termed BiTEs, enhance the patient's immune response to tumors by retargeting T cells to tumor cells. BiTEs are constructed of two single chain variable fragments (scFv) connected in tandem by a flexible linker. One scFv binds to a T cell-specific molecule, usually CD3, while the second scFv binds to a tumor-associated antigen. This

[17] LAG3 Lymphocyte-activation protein 3 belongs to Ig superfamily and contains 4 extracellular Ig-like domains. The LAG3 gene contains 8 exons. The sequence data, exon/intron organization, and chromosomal localization all indicate a close relationship of LAG3 to CD4. (see https://www.ncbi.nlm.nih.gov/gene/3902) CTLA-4 is a checkpoint protein and is targeted by many Abs in immunotherapy. his gene is a member of the immunoglobulin superfamily and encodes a protein which transmits an inhibitory signal to T cells. The protein contains a V domain, a transmembrane domain, and a cytoplasmic tail. Alternate transcriptional splice variants, encoding different isoforms, have been characterized. The membrane-bound isoform functions as a homodimer interconnected by a disulfide bond, while the soluble isoform functions as a monomer. (see https://www.ncbi.nlm.nih.gov/gene/1493)

structure and specificity allow a BiTE to physically link a T cell to a tumor cell, ultimately stimulating T cell activation, tumor killing and cytokine production. BiTEs have been developed that target several tumor-associated antigens for a variety of both hematological and solid tumors. Several BiTEs are currently in clinical trials for their therapeutic efficacy and safety. This review examines the salient structural and functional features of BiTEs as well as the current state of their clinical and preclinical development….

The concept of using T cell retargeting for cancer therapy stretches back to the 1970s. Unlike macrophages, dendritic cells, and other accessory cells, T cells are present in copious numbers, expand rapidly upon activation, give robust and durable cytotoxic responses, and have the potential to generate immunologic memory. Furthermore, T cells have been found to attack tumors from the outside as well as infiltrating into the tumor. These features make T cells optimal therapeutic effectors for cancer. T cell redirection does suffer one significant challenge, which is the requirement of a second stimulatory signal to achieve full T cell activation and prevent anergy. Multiple bispecific formats have been developed to meet or circumvent this requirement.

Then Abbas et al also have noted:

Bispecific T cell engagers (BiTEs) facilitate the targeting of host T cells of any specificity to attack tumor cells. These reagents are recombinant antibodies engineered to express two different antigen binding sites, one specific for a tumor antigen and the second specific for a T cell surface molecule, usually CD3. In many of these antibodies, each antigen binding site is composed of a single chain variable fragment containing Ig heavy and light chain variable domains, similar to the CARs described earlier.

The presumed mechanism of action of BiTEs, based on in vitro studies, is the formation of immune synapses between the tumor cells and the T cells and the activation of the T cells by CD3 crosslinking. A CD19-specific BiTE is approved for treatment of acute lymphocytic leukemia. BiTEs specific for many other tumor antigens have been developed, including CD20, EpCAM, Her2/neu, EGFR, CEA, folate receptor, and CD33, and are at various stages of preclinical and clinical trials.
As Ross et al note:

For targets that are homogenously expressed, such as CD19 on cells of the B lymphocyte lineage, immunotherapies can be highly effective. Targeting CD19 with blinatumomab, a CD19/CD3 bispecific antibody construct (BiTE®), or with chimeric antigen receptor T cells (CAR-T) has shown great promise for treating certain CD19-positive hematological malignancies.

In contrast, solid tumors with heterogeneous expression of the tumor-associated antigen (TAA) may present a challenge for targeted therapies. To prevent escape of TAA negative cancer cells, immunotherapies with a local bystander effect would be beneficial. As a model to investigate BiTE®-mediated bystander killing in the solid tumor setting, we used epidermal growth factor receptor (EGFR) as a target. We measured lysis of EGFR-negative populations in vitro and in vivo when co-cultured with EGFR-positive cells, human T cells and an EGFR/CD3 BiTE®

antibody construct. Bystander EGFR-negative cells were efficiently lysed by BiTE®-activated T cells only when proximal to EGFR-positive cells.

Our mechanistic analysis suggests that cytokines released by BiTE®-activated T-cells induced upregulation of ICAM-1 and FAS on EGFR-negative bystander cells, contributing to T cell induced bystander cell lysis.

Namely the BITE approach is to create using an Ab a molecule which is CD3 on one end and say CD19 on the other and use this to cover a target and then to attract a T cell. In some ways this is akin to CAR-T where we place the receptor to the target on a T cell, here we use a T cell and attach the target to a known receptor on a T cell.

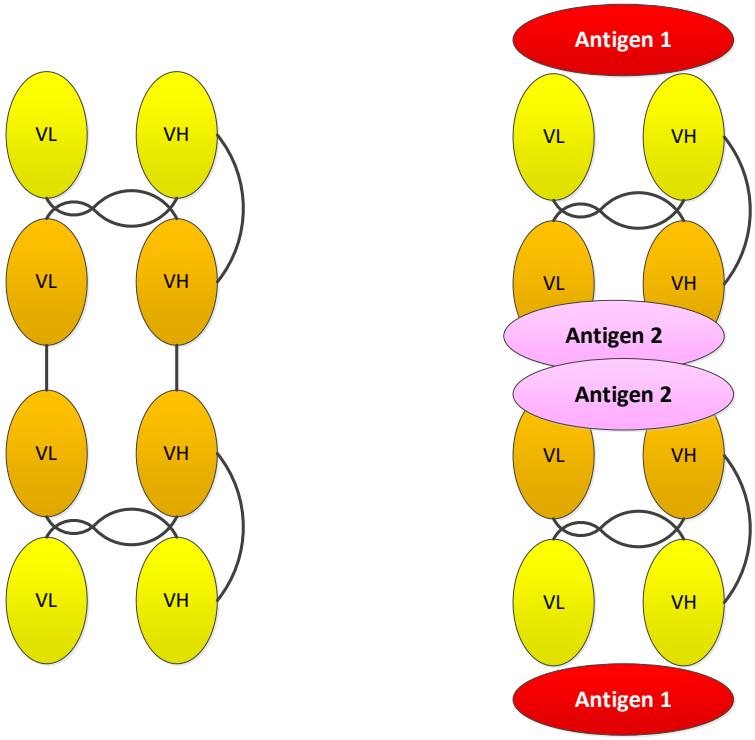
Furthermore, Zahavi and Weiner have recently noted:

Recently, the most successful mAb-based strategies have moved away from targeting tumor antigens and instead focused on targeting immune cells in order to enhance their anti-tumor capabilities. One of the first mAb approaches to stimulate T cell anti-tumor immunity was the development of bispecific T Cell Engager (BiTE) antibodies that both target a tumor antigen such as CD19 and the activating receptor, CD3, on T cells. BiTEs combine direct targeting of tumor cells with recruitment of cytotoxic T cells into the tumor microenvironment and led to tumor regressions even when administered at doses three orders of magnitude less than the parent mAb alone. The CD19-CD3 BiTE blinatumomab conferred significant clinical benefit to acute lymphoblastic leukemia patients and was FDA approved in 2017.

Clinical trials are currently underway using BiTEs generated from the widely used anti-HER2 and anti-EGFR mAbs trastuzumab and cetuximab. Other mAb approaches seek to enhance T cell specific immunity against tumor cells by stimulating activating receptors such as 4-1BB, OX40, CD27, CD40, and ICOS. Agonist antibodies towards CD40 stimulate antigen presentation by dendritic cells and mAbs to OX40 and 4-1BB activate T cells while simultaneously dampening the activity of inhibitory T regulatory cells (Tregs) . mAbs designed to stimulate these activating receptors are in various stages of clinical trials both alone and in combination with other immunotherapy approaches. Additional mAbs that deplete inhibitory Tregs directly, such as daclizumab, which targets CD25 on Tregs, are also undergoing clinical trials

2. TandAb

TandAb is s homodimer consisting of four scFv motifs with linkers. Shown below it is a complex protein structure with multiple Ag binding sites.



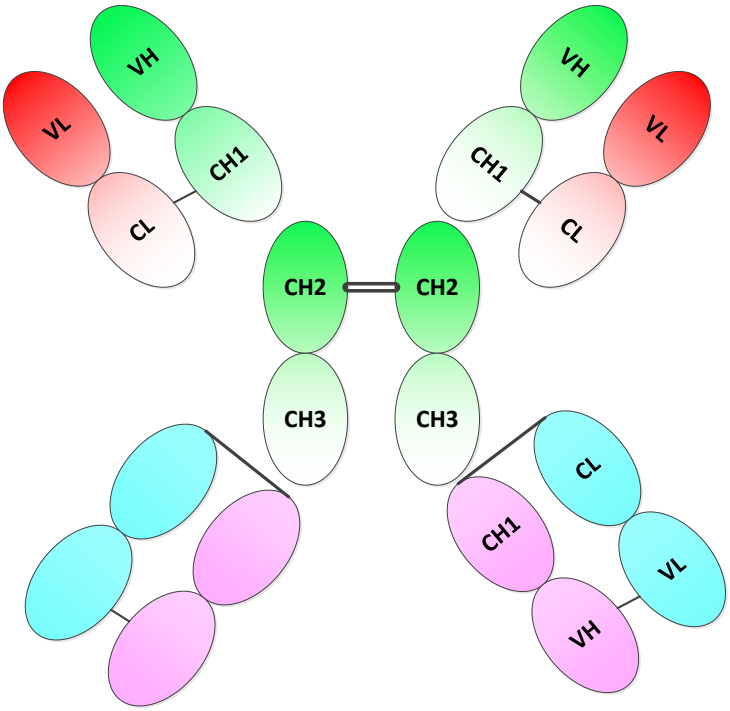
The TandAb form has been developed to block CD3 and CD19.

4.1.1.3 PreClinical

We now present a mix of preclinical polyAb.

1. biAbFabL

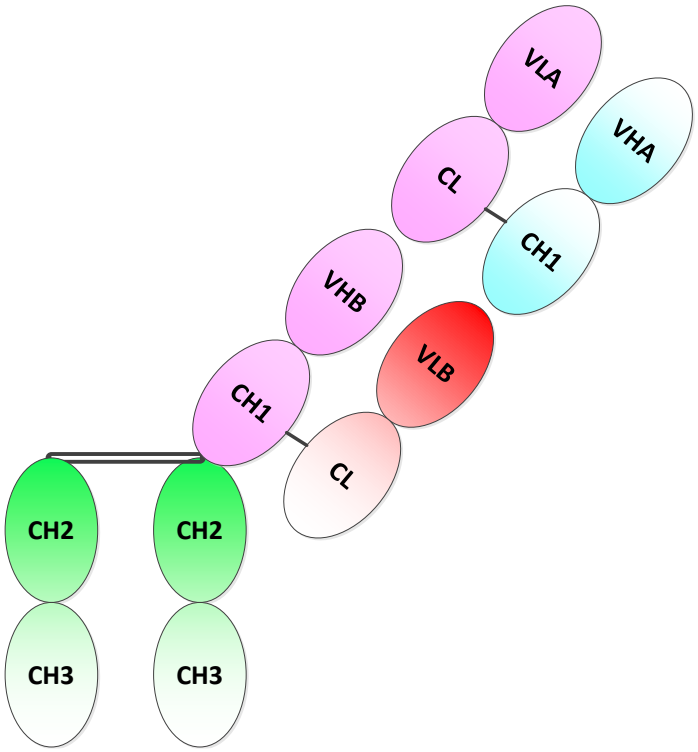
The biAbFabL is shown below and is composed of two Fab domains with a central C domain. Thus, the Ab is tetravalent.



The targets for some current developments have been IL-17 and IL-23 inhibition. These target a multiple set of inflammatory disease such as IBD, Chron's, MS and psoriasis.

2. MAT-Fab

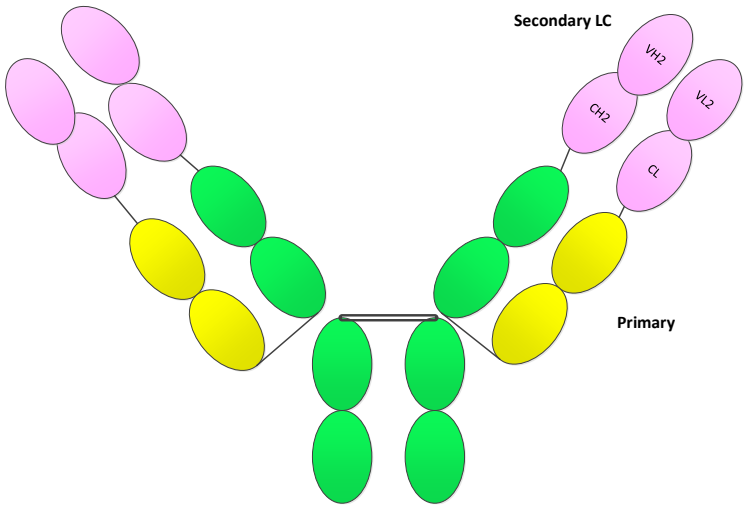
MAT-Fab is a complex tetrameric protein having four protein sections as shown below.



As with previous ones it targets T cells and also NK cells and macrophages. Some targets are CD3 on T cells as well as CD20 on specific cancer cells.

1. Tandem Forms

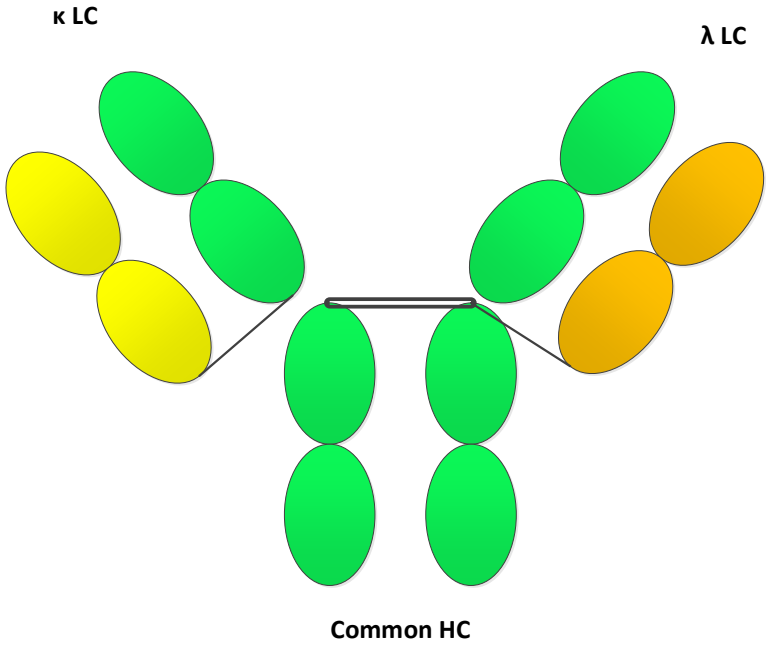
The Tandem form is a “Y-shaped” bispecific antibody format. It closely resembles that of standardized IgG antibodies, and, while being equipped with an Fc region and Fab regions, distinguished itself by having two sets of two Fab regions of different specificity linked in tandem in the Figure below. This enabled each form to retain moderately high to high binding affinity to both antigens. They are hence functional homodimeric tetravalent bispecific antibodies



The therapeutic design focuses on Toll Like Receptors, TLR, especially TLR 2 and TLR 4.

2. κλ antibody

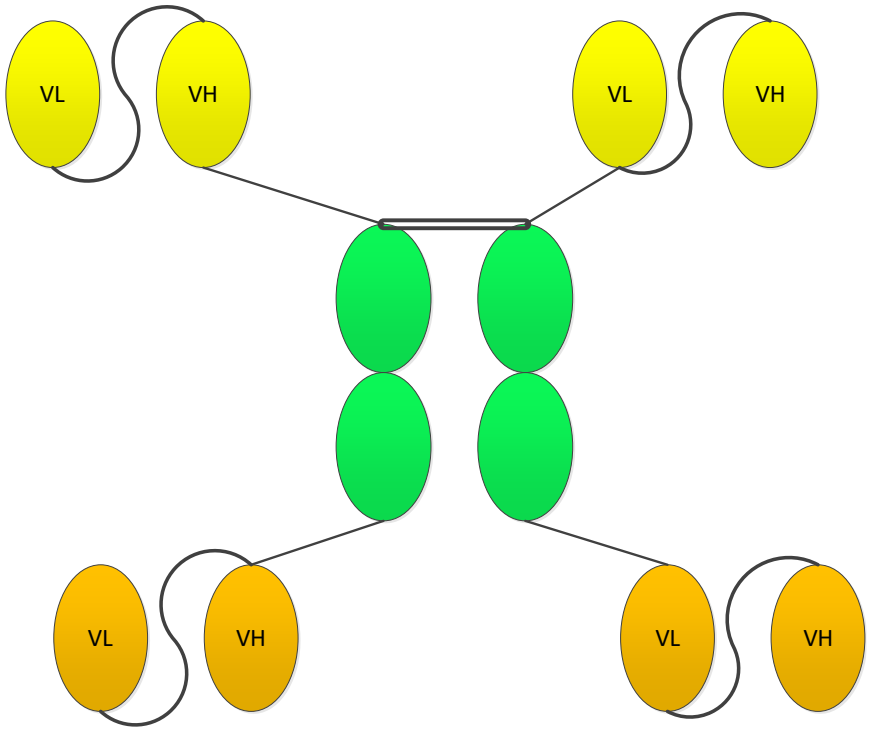
The antibody in this configuration is IgG like in structure except that it has two distinct Fab regions. These two light chains give bi-specific capability.



The therapeutic target is CD47 which appears on tumors and prevent T cell action. It blocks that target. It also blocks CD19

1. ADAPTIR

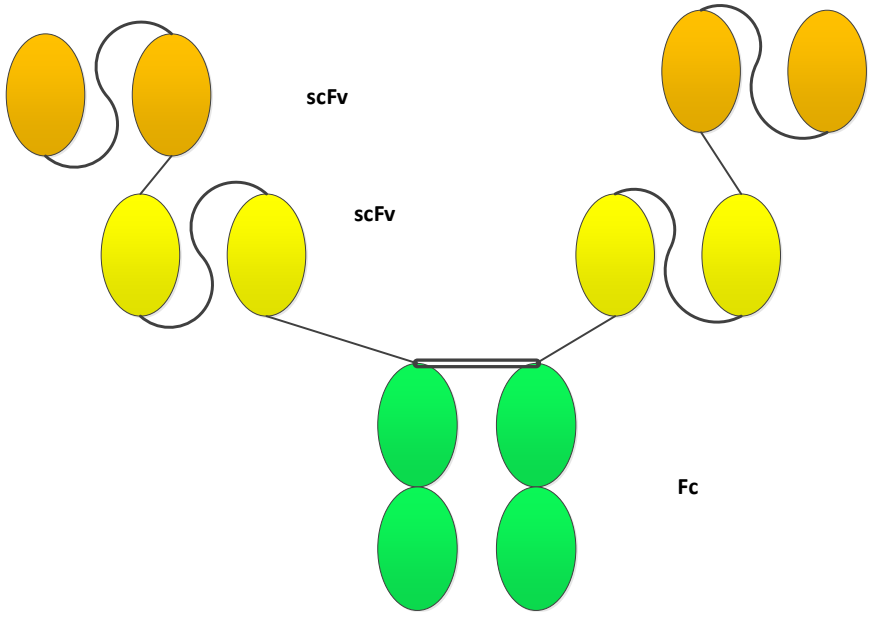
ADAPTIR as a bispecific antibody is comprised of an Fc region and four binding domains with two different specificities. The four binding domains are scFvs and attached in pairs at the amino and carboxyl ends of the Fc region. Thus, the Fc region has two binding domains at each end for binding two different antigens respectively, making it a tetravalent homodimeric bispecific antibody



The therapeutic target is a tumor necrosis factor 4-IBB and a tumor associated antigen 5T4. Targeting these two molecules with a bispecific antibody will promote potent tumor-directed immune T cell activation which makes ALG.APV-527 a potential drug for treatment of cancer.

2. BiIA-SG

This structure is a bispecific immunoadhesin bs-BnAb called BiIA-SG. It is an engineered immunoadhesin, which is an antibody-like molecule. It tetravalently binds to the two antigens via four scFvs fused to an IgG Fc region. It lacks the two CH1 domains that are native to the heavy chains of the IgG structure. The structure of the single gene-encoded BiIA-SG molecule is constructed using a gene tandem fusion method. This results in a structurally unique molecule with four scFv binding domains, two targeting HIV-1 gp120 receptor and 2 targeting human T cell CD4 receptor. The existing of two scFv for gp120 results in a significant higher binding affinity comparison to having only one.



This has been designed to treat HIV infections.

4.1.2 Tri-specifics

Tri-specific antibodies is the next step in Ab enhancements. For example, if two are good are three better? In a paper by Garfall and June they note:

Antibodies with specificity for one target — called monoclonal antibodies — were the first cancer immunotherapy to achieve widespread clinical use. The therapeutic potency of antibodies can be amplified by engineering them to recognize two distinct molecular targets (termed antigens). These bispecific antibodies can simultaneously bind to cancer cells and immune cells called T cells, and this dual binding directs the T cell to unleash its cell-killing power towards the cancer cell.

Writing in Nature Cancer, Wu et al now report the development of a trispecific antibody, one that has three targets: a cancer cell, a receptor that activates T cells, and a T-cell protein that promotes long-lasting T-cell activity against the cancer cell…. the development of a human antibody that is engineered to bring an immune cell called a T cell into close proximity with a type of cancer cell called a myeloma cell and to boost the T cell's anticancer response.

This trispecific antibody binds three targets:

(i) the protein CD38 on a myeloma cell, and

(ii) the protein CD28 and the

(iii) protein complex CD3 on a T cell.

CD3 is part of the T-cell receptor (TCR), which recognizes abnormal cells by binding molecules called antigens. The binding of CD3 by the antibody drives T-cell activation (without requiring antigen recognition by the TCR), which leads to the killing of the myeloma cell and the production and release of toxic cytokine molecules.

Binding of CD28 by the antibody drives expression of the protein Bcl-xL. Bcl-xL blocks T-cell death, which might otherwise occur if there was prolonged TCR activation in the absence of CD28 stimulation by the antibody.

This is a three-way binding, a bi-specific plus one. As Guo et al have noted:

Oncolytic viruses (OVs) are potent anti-cancer biologics with a bright future, having substantial evidence of efficacy in patients with cancer. Bi- and tri-specific antibodies targeting tumor antigens and capable of activating T cell receptor signaling have also shown great promise in cancer immunotherapy. In a cutting-edge strategy, investigators have incorporated the two independent anti-cancer modalities, transforming them into bi- or tri-specific T cell engager (BiTE or TriTE)-armed OVs for targeted immunotherapy. Since 2014, multiple research teams have studied this combinatorial strategy, and it showed substantial efficacy in various tumor models. Here, we first provide a brief overview of the current status of oncolytic virotherapy and the use of multi-specific antibodies for cancer immunotherapy.

We then summarize progress on BiTE and TriTE antibodies as a novel class of cancer therapeutics in preclinical and clinical studies, followed by a discussion of BiTE- or TriTE-armed OVs for cancer therapy in translational models. In addition, T cell receptor mimics (TCRm) have been developed into BiTEs and are expected to greatly expand the application of BiTEs and BiTE-armed OVs for the effective targeting of intracellular tumor antigens. Future applications of such innovative combination strategies are emerging as precision cancer immunotherapies.

Thus tri-specifics add an additional dimension to the targeting. This may increase specificity and reduce an adverse reactions, yet that is yet to be fully understood. What is clear is that the more we know about a specific cancer molecule the more we can target it and the less damage we may incur in the process.

As Runcie et al note:

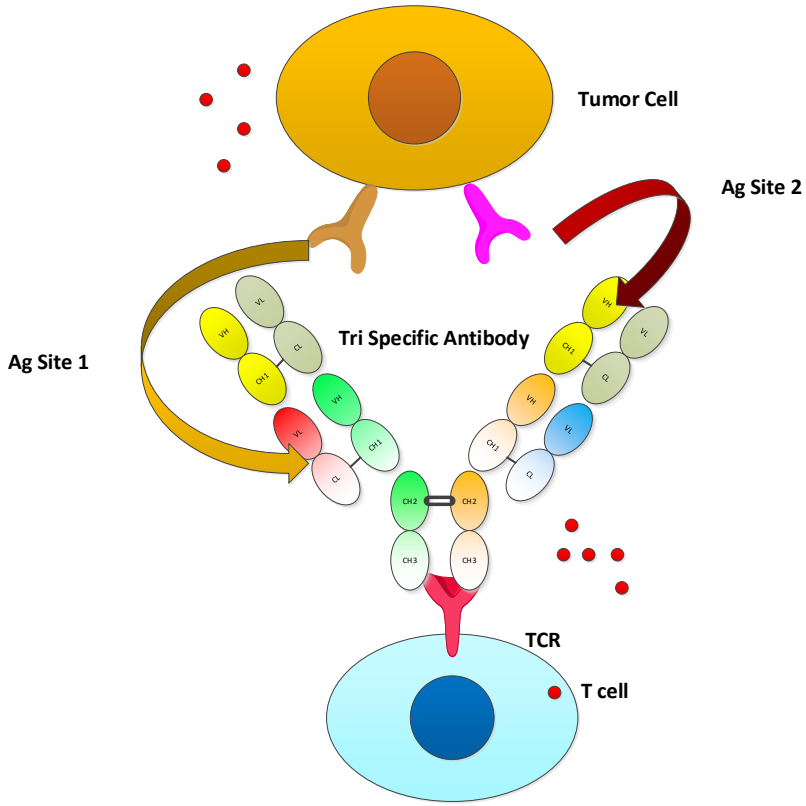
Innovative techniques to harness natural killer cell in immunotherapy have introduced the concept of ***bi-specific killer cell engagers (BiKEs) and tri-specific killer cell engagers (TriKEs). BiKEs are created by the fusion of a single chain variable fragment (Fv) against CD 16 (antigen on natural killer cells) and a single-chain Fv against a tumor associated antigen.***

TriKEs are a combination of a single-chain Fv against CD16 and two tumor associated antigens*.*

These molecules directly trigger NK cell activation through CD 16 amplifying NK cell cytolytic activity and cytokine production against various tumor cell antigen targets. These drugs are currently being investigated in preclinical studies and safety remains a concern with the potential to trigger cytokine cascades …

Even though most polyspecific antibodies have two binding sites (bispecific), there are many new molecules with three or four binding sites. For example, Castoldi et al., have recently developed a tetravalent Fc containing antibody (tetramab) directed against HER1, HER3, c-MET and IGF1R with enhanced antitumor effects in a preclinical model

Runcie et al depict a typical tri-specific as below:



4.1.3 Antibodies and PSMA

There have been several recent efforts focusing on Abs and PSMA. Heitmann et al is one of the more recent and they report:

Prostate cancer is the second most common cancer in men worldwide, with estimated 1100000 cases and 307000 deaths in 2012. Androgen-deprivation therapy is standard of care first-line therapy of advanced prostate cancer. However, frequently prostate carcinoma develops resistance to first-line therapy.

Notably, most drugs established for treatment of these castration-resistant prostate carcinomas (CRPCs) (eg, abiraterone acetate, enzalutamide) still act on the androgen axis.

Applied prior to or after treatment with chemotherapeutic agents (eg, docetaxel, cabazitaxel), these therapies slow down disease progression and improve survival to a moderate extent.

Abiraterone resulted in a median overall survival (OS) benefit of 4.6 months post-docetaxel and of 4.4 months in chemotherapy-naive patients.

Enzalutamide resulted in a median OS benefit of 4.8 months post-chemotherapy.

The chemotherapeutic agents docetaxel and cabazitaxel resulted in a median OS benefit of 2.4months.

In case of progression/relapse, for example, abiraterone can be used after enzalutamide or after docetaxel and vice versa. Importantly, the best sequence of treatments has not been finally established, and any drug employed after the third line of treatment is associated with only limited clinical benefit. Novel strategies have to be developed to address the medical need of this patient population.

Of particular interest in this context are strategies to target the prostate-specific membrane antigen (PSMA), which is expressed, at least to some extent, in almost all patients (up to 98%) with a highly tumour-restricted expression pattern.

Targeted radiotherapy approaches using for example, Lutetium-177-PSMA8 showed efficacy and a tolerable toxicity profile on treatment of patients with metastatic disease. However, the duration of achieved responses is limited, and many patients do not at all benefit from this treatment option. Meanwhile, immunotherapy has become a mainstay of oncological treatment. Available strategies comprise immune checkpoint blocking antibodies (eg, nivolumab, pembrolizumab) that are approved for treatment of various solid tumours including non-small cell lung cancer, melanoma and renal cell carcinoma.

However, these checkpoint inhibitors have shown only limited efficacy in prostate cancer. Other *successful antibody-based strategies that mobilize T cells against cancer comprise bispecific antibodies (bsAbs) and chimeric antigen receptor T (CART) cells. The first stimulate the T cell receptor/CD3-complex with their effector part after binding their target antigen on tumour cells. The latter are functionally closely related to the bsAb, as CART cells can be considered as genetically modified T cells with an integrated bsAb (CD3 signalling unit anchored in the T cell).*

The most advanced reagent in the class of bsAbs is the CD19xCD3 bsAb blinatumomab (Amgen) approved for treatment of acute lymphoblastic leukaemia in the bispecific single-chain (BiTE) format. Like bsAb, CART cells are mainly established for treatment of lymphoid malignancies, and so far, both, bsAbs and CART cells, are less effective if applied against solid tumours compared with haematological malignancies. In our view, a major problem is the lacking accessibility of solid tumours for the effector cells, be it for CART cells or bsAb-stimulated T cells.

Sustained therapeutic success of both, bsAb and CART cells are further limited by the severe side effects, in particular the potentially lethal ***cytokine-release syndrome (CRS)*** *as most important group toxicity. At present, if CRS occurs, it is treated with interleukin-6 receptor (IL-6R) blockade using tocilizumab.20 We have developed an optimised bsAb with PSMAxCD3 specificity (CC-1) that, on application after pre-emptive IL-6R blockade holds promise to overcome the above-described limitations.*

Of note, ***targeting PSMA with bsAbs not only holds promise to potentially induce more pronounced 'immediate effects' compared with PSMA targeted radiotherapy, but may also stimulate immunological memory and thus mediate long-term efficacy.*** *In our first in human (FIH) study reported here, we evaluate CC-1 in patients with metastatic CRPC to determine overall safety and tolerability, as well as the maximum tolerated dose (MTD) and first signs of efficacy*

As Niaz et al have noted:

Cancer cells can be selectively targeted by identifying and developing antibodies to specific antigens present on the cancer cell surface. Cytotoxic agents can be conjugated to these antibodies that bind to these cell surface antigens in order to significantly increase the therapeutic index of whichever cytotoxic agent is utilized. This approach of conjugating the cytotoxic drugs to antibodies to target specific surface antigens enhances the anti-tumor activity of antibodies and improves the tumor-to-normal tissue selectivity of chemotherapy.

Critical parameters in the development of these antibody-drug conjugates include:

1) selection of most appropriate antigen,

2) the ability of an antibody to be internalized after binding to the antigen,

3) cytotoxic drug potency and

4) stability of the antibody-drug conjugate.

For prostate cancer, prostate-specific membrane antigen (PSMA, also known as folate hydrolase-1) is the most validated theragnostic target to date. PSMA is overexpressed on the prostate cancer cell surface, which makes it an even better target for selective drug delivery through conjugated antibodies. Here, we review the PSMA-based antibody-drug conjugates for metastatic castration-resistance prostate cancer (mCRPC)….

MLN2704 is an immunoconjugate between maytansinoid-1 (DM 1) and the humanized J591 antibody (MLN591), and it is designed to deliver the maytansinoid antimicrotubule agent, DM 1, directly to prostate-specific membrane antigen (PSMA) expressing cells. J591 (MLN591) is an anti-PSMA monoclonal antibody, and it has the property of becoming internalized once bound to the extracellular domain of PMSA…

Antibody-drug conjugate, as a concept, is very promising for clinical investigation and remains an active area of research. Although MLN2704 clinical trials resulted in an unfavorable safety profile due to instability of the antibody-drug conjugate, it validated PSMA as an important immunoconjugate target. With a di-peptide linker, PSMA-ADC was associated with lower but still significant rates of neurotoxicity, again due to deconjugation. As novel and more effective linker agents are developed and enter clinical investigation, adverse events will decrease, while the efficacy of antibody-drug conjugates will improve.

Ab can also be used to target PSMA but for the delivery of alternative therapeutics. As Lucio et al have noted:

The ability of carbon nanohorns (CNHs) to cross biological barriers makes them potential carriers for delivery purposes.

In this work, we report the design of a new selective antibody–drug nanosystem based on CNHs for the treatment of prostate cancer (PCa). In particular, cisplatin in a prodrug form and the monoclonal antibody (Ab) D2B, selective for PSMA+ cancer cells, have been attached to CNHs due to the current application of this antigen in PCa therapy.

The hybrids Ab–CNHs, cisplatin–CNHs and functionalised-CNHs have also been synthesized to be used as control systems.

The efficacy and specificity of the D2B–cisplatin–CNH conjugate to selectively target and kill PSMA+ prostate cancer cells have been demonstrated in comparison with other derivatives. The developed strategy to functionalise CNHs is fascinating because it can allow the fine tuning of both drug and Ab molecules attached to the nanostructure in order to modulate the activity of the nanosystem. Finally, the herein described methodology can be used for the incorporation of almost any drugs or Abs in the platforms in order to create new targeted drugs for the treatment of different diseases …

Abs recognizing some tumour associated antigens (TAAs) are currently applied ‘‘naked’’, conjugated to radiochemicals or to chemotherapeutic drugs in the clinics.6 They have also been used to improve the selectivity of carbon nanomaterials due to their easy conjugation to the nanostructures, the high affinity and stability,7–9 showing promising results in tumour diagnosis and therapy.

Prostate cancer (PCa) is the most common cancer in man in industrialized countries and it can be often treated successfully when diagnosed in the early stages; local and regional stages show a 5 year relative survival rate nearly 100%.11 Unfortunately patients where cancers have spread to distant lymph nodes, bones, or other organs show a drastic decrease of 5 year relative survival rate (i.e. survival rate of 28%), although some surgical, chemotherapeutic or radiotherapeutic treatments (i.e. alone or in combination) are performed. Therefore new therapeutical approaches are needed and, among these, the targeted approaches based on the recognition of cell associated tumour antigens are promising …

A new series of hybrid materials composed of carbon nanohorns as delivery vehicles (Ab–CNH, drug–CNH, Ab–drug–CNH and double functionalised-CNH) have been synthesized and fully characterized. In particular, cisplatin in a prodrug form and a specific D2B antibody for PSMA+ prostate cancer cells have been attached. Different biological experiments have demonstrated the selective binding and uptake of the conjugates with antibody (Ab–CNH and Ab–drug–CNH) on PSMA+ prostate cancer cells. Finally, the selectivity of the derivative Ab–drug–CNH on PSMA+ prostate cancer cells has made possible their selective killing versus PSMA prostate cancer cells.

This property is enhanced when the nanosystems are shielded with BSA. In conclusion, we have demonstrated the better ability of f11-CNH to selectively kill PSMA+ cancer cells in comparison with the other synthesized CNH hybrids. Furthermore, this new system offers great potentiality due to the possibility of modifying the type and degree of functionalization.

This allows the variation of the quantity of drug or antibody attached to the nanostructure in order to play with the killing efficacy. Similarly, the method is useful to attach different drugs or antibodies opening the way to the treatment of other diseases.

Bi specific Ab have been developed targeting PSMA as well as a second target, here CD3. Leconet et al have thus noted:

Small therapeutic proteins represent a promising novel approach to treat cancer. Nevertheless, their clinical application is often adversely impacted by their short plasma half-life. Controlled long-term delivery of small biologicals has become a challenge because of their hydrophilic properties and in some cases their limited stability.

Here, an in situ forming depot-injectable polymeric system was used to ***deliver BiJ591, a bispecific T-cell engager (BiTE) targeting both prostate-specific membrane antigen (PSMA) and the CD3 T-cell receptor in prostate cancer****.*

BiJ591 induced T-cell activation, prostate cancer–directed cell lysis, and tumor growth inhibition. The use of diblock (DB) and triblock (TB) biodegradable polyethylene glycol–poly(lactic acid; PEG-PLA) copolymers solubilized in tripropionin, a small-chain triglyceride, allowed maintenance of BiJ591 stability and functionality in the formed depot and controlled its release. In mice, after a single subcutaneous injection, one of the polymeric candidates, TB1/DB4, provided the most sustained release of BiJ591 for up to 21 days.

Moreover, the use of BiJ591-TB1/DB4 formulation in prostate cancer xenograft models showed ***significant therapeutic activity in both low and high PSMA****–expressing tumors, whereas daily intravenous administration of BiJ591 was less efficient. Collectively, these data provide new insights into the development of controlled delivery of small therapeutic proteins in cancer….*

In this study, the ***scFv of the anti-prostate specific membrane antigen (PSMA) J591 antibody*** *was used to design* ***a BiTE antibody****, designated BiJ591, targeting PSMA and CD3. After demonstrating the specific cytotoxicity of BiJ591 to PSMApositive prostate cancer models in vitro and in vivo, the protein was formulated in a long-acting injectable technology drugdelivery*

system composed of diblock (DB) and triblock (TB) biodegradable polyethylene glycol (PEG)–poly(lactic acid; PLA) copolymers solubilized in tripropionin, a small-chain triglyceride. The stability and functionality of BiJ591 throughout the formulation process were demonstrated, and a single dose of a BiJ591 polymeric formulation injected subcutaneously significantly improved the apparent elimination half-life as well as the in vivo antitumor activity of the bispecific antibody in cancer xenograft models in comparison with a daily intravenous administration….

In conclusion, an injectable in situ biodegradable polymerbased protein delivery system was successfully designed to prolong the in vivo elimination half-life and the therapeutic effect of a small bispecific T-cell engager targeting PSMA in prostate cancer.

The polymeric technology preserves the stability and functionality of the protein and the composition of the polymers can modulate the release profile in vitro and in vivo. Very encouragingly, a significant decrease in tumor growth was observed in animal models upon the administration of the formulated bispecific antibody. This technology represents a promising therapeutic approach and could be transposed to other cancer types using similar bispecific antibodies scaffolds able to target different tumor markers.

4.2 CAR-T CELLS

CAR-T cells have been used now for almost a decade[18]. They are structured T cell that target specific proteins or antigen like elements. As Montagner et al note:

Prostate cancer (PCa) has become the most common cancer among males in Europe and the USA. Adoptive immunotherapy appears a promising strategy to control the advanced stages of the disease by specifically targeting the tumor, in particular through chimeric antigen receptor T (CAR-T) cell therapy. Despite the advancements of CAR-T technology in the treatment of hematological malignancies, solid tumors still represent a challenge. To overcome current limits, other cellular effectors than T lymphocytes are under study as possible candidates for CAR-engineered cancer immunotherapy.

A novel approach involves the NK-92 cell line, which mediates strong cytotoxic responses against a variety of tumor cells but has no effect on non-malignant healthy counterparts.

Here, we report a novel therapeutic approach against PCa based on engineering of NK-92 cells with a CAR recognizing the human prostate-specific membrane antigen (PSMA), which is overexpressed in prostatic neoplastic cells. More importantly, the potential utility of NK-92/CAR cells to treat PCa has not yet been explored. Upon CAR transduction, NK-92/CAR cells acquired high and specific lytic activity against PSMA-expressing prostate cancer cells in vitro, and also underwent degranulation and produced high levels of IFN-γ in response to antigen recognition. Lethal irradiation of the effectors, a safety measure requested for the clinical application of retargeted NK-92 cells, fully abrogated replication but did not impact on phenotype and short-term functionality.

[18] https://www.researchgate.net/publication/309419224_CAR_T_Cells_and_Cancer

PSMA-specific recognition and antitumor activity were retained in vivo, as adoptive transfer of irradiated NK-92/CAR cells in prostate cancer-bearing mice restrained tumor growth and improved survival. Anti-PSMA CAR-modified NK-92 cells represent a universal, off-the-shelf, renewable, and cost-effective product endowed with relevant potentialities as a therapeutic approach for PCa immunotherapy

4.2.1 CAR-T Cells Redux

CAR-T cells are essentially engineered T cells, specifically cytotoxic T lymphocytes, CTL, engineered to target specific cells such as those in various hematopoietic cell lines. such as leukemias and lymphomas. There is no fundamental reason that they cannot be used for solid tumors but there are certain operational barriers which must be overcome.

As Kershaw et al note:

There are two main types of antigen receptors used in genetic redirection.

The first utilizes the native alpha and beta chains of a TCR specific for tumor antigen.

The second is termed a chimeric antigen receptor (CAR), which is composed of an extracellular domain derived from tumor-specific antibody, linked to an intracellular signaling domain. Genes encoding these receptors are inserted into patient's T cells using viral vectors to generate tumor reactive T cells….

The specificity of CARs is derived from tumor-specific antibodies, which are relatively simple to generate through immunization of mice. Recombinant techniques can be used to humanize antibodies, or mice expressing human immunoglobulin genes can be used to generate fully human antibodies. Single-chain variable fragments of antibodies are used in the extracellular domain of CARs, which are joined through hinge and transmembrane regions to intracellular signaling domains.

As Miller and Sadelain note:

The advent of gene transfer technologies, in particular those enabling the transduction of human T lymphocytes using gibbon ape leukemia virus envelope-pseudotyped g-retroviral vectors, created new opportunities for immune intervention based on T cell engineering. Patients' T cells, easily accessible in peripheral blood, can be genetically instructed to target tumors by transduction of receptors for antigen, utilizing either the physiological TCR or synthetic receptors now known as CARs.

Both approaches have shown clinical successes, particularly in melanoma, targeting NYESO1, and in acute lymphoblastic leukemia, CARs are artificial, composite receptors for antigen that integrate principles of B cell and T cell antigen recognition. They are particularly attractive in that they elude human leucocyte antigen (HLA) restriction and are thus applicable to all patients irrespective of their HLA haplotypes, unlike TCRs. CARs may also overcome HLA downregulation by tumors, which deprives T cells of a ligand for their endogenous TCR.

The critical function of CARs is, however, not to merely target the T cells to a tumor antigen, but to enhance T cell function. Thus, effective CARs further integrate principles of T cell costimulation and provide a broad spectrum of functional enhancements acquired by directly soliciting selected costimulatory pathways

Juillerat et al note:

Adoptive immunotherapy using engineered T-cells has emerged as a powerful approach to treat cancer. The potential of this approach relies on the ability to redirect the specificity of T cells through genetic engineering and transfer of chimeric antigen receptors (CARs) or engineered TCRs1. Numerous clinical studies have demonstrated the potential of adoptive transfer of CAR T cells for cancer therapy but also raised the risks associated with the cytokine-release syndrome (CRS) and the "on-target off-tumor" effect.

To date, few strategies have been developed to pharmacologically control CAR engineered T-cells and may rely on suicide mechanisms. Such suicide strategies leading to a complete eradication of the engineered T-cells will result in the premature end of the treatment. Consequently, implementing non-lethal control of engineered CAR T-cells represents an important advancement to improve the CAR T-cell technology and its safety.

Small molecule based approaches that rely on dimerizing partner proteins have already been used to study, inter alia, the mechanism of T-cell receptor triggering15. Very recently, Lim and colleagues have adapted this approach to control engineered T-cells through the use of a multichain receptor.

Here, we describe a strategy to create a switchable engineered CAR T-cells. Our approach is based on engineering a system that is directly integrated in the hinge domain that separate the scFv from the cell membrane. In addition, we chose to implement this strategy in a novel CAR architecture that relies on the FceRI receptor scaffold.

The particularity of this design resides in the possibility to split or combine different key functions of a CAR such as activation and costimulation within different chains of a receptor complex, mimicking the complexity of the TCR native architecture. In this report, we showed that the hinge engineering approaches allowed to turn a T-cell endowed with an engineered CAR from an off-state to an on-state.

By controlling the scFv presentation at the cell surface upon addition of the small molecule, our system allowed to further induce the cytolytic properties of the engineered T-cell. Overall, this non-lethal system offers the advantage of a "transient CAR T-cell" for safety while letting open the possibility of multiple specific cytotoxicity cycles using a small molecule drug.

Principles of T Cell Engineering and CAR Design

(A) Integration of B cell and T cell antigen recognition principles in the design of CARs. The heavy and light chain chains, which are components of the B cell receptor and Igs, are fused to

the T-cell-activating z chain of the TCR-associated CD3 complex to generate non-MHC restricted, activating receptors capable of redirecting T cell antigen recognition and cytotoxicity.

(B and C) Integration of T cell activation and costimulation principles in dual signaling CARs designed to enhance T cell function and persistence in addition to retargeting T cell specificity. In

(B), the physiological abTCR associated with the CD3 signaling complex is flanked by the CD28 costimulatory receptor.

(C) shows a prototypic second-generation CAR, which comprises three canonical components: an scFv for antigen recognition, the cytoplasmic domain of the CD3z chain for T cell activation, and a costimulatory domain to enhance T cell function and persistence. Unlike the abTCR/CD3 complex, which comprises g, d, ε, and z signaling chains and is modulated by a multitude of costimulatory receptors, CARs possess in a single molecule the ability to trigger and modulate antigen-specific T cell functions.

4.2.2 Generational Architecture

There are currently three generations of CAR T cell design. We examine each here. As Cartellieri et al note:

In an attempt to extend the recognition specificity of T lymphocytes beyond their classical MHC-peptide complexes, a gene-therapeutic strategy has been developed that allows redirecting T cells to defined tumor cell surface antigens. This strategy uses both the cellular and humoral arm of the immune response by assembling an antigen-binding moiety, most commonly a single chain variable fragment (scFv) derived from a monoclonal antibody, together with an activating immune receptor.

Once this artificial immune receptor is expressed at the surface of a modified T lymphocyte, upon binding of the scFv to its antigen an activating signal is transmitted into the lymphocyte, which in turn triggers its effector functions against the target cell (Figure 2). In the first attempts to reconfigure T cells with antibody specificity the variable parts of the TCR α and β chains were replaced with scFv fragments derived from monoclonal antibodies. These hybrid T-cell receptors were functionally expressed and recognized the corresponding antigens in a non-MHC-restricted manner.

As a consequence of the finding, that CD3ζ chain signaling on its own is sufficient for T-cell activation, the first "true" chimeric single-chain receptors were created by fusing a scFv directly to the CD3ζ chain. At that time this concept was called the "T body approach". Nowadays these types of artificial lymphocyte signaling receptors are commonly referred to as chimeric immune receptors (CIRs) or chimeric antigen receptors (CARs).

The use of CARs to redirect T cells specifically against TAA-expressing tumor cells has a number of theoretical advantages over classical T-cell-based immunotherapies. In contrast to the long-lasting procedure of in vitro selection, characterization, and expansion of T-cell clones

with native specificity for MHC tumor peptide complexes, genetic modification of polyclonal T-cell populations allows to generate TAA-specific T cells in one to two weeks. Engraftment with CARs enables T cells to MHC-independent antigen recognition; thus, major immune escape mechanisms of tumors such as downregulation of MHC molecules are efficiently bypassed.

Furthermore, proliferation and survival of modified T cells can be improved by the implementation of a multitude of signaling domains from different immune receptors in a single CAR

4.2.3 First Generation

Following Cartellieri et al we note regarding all three generations that:

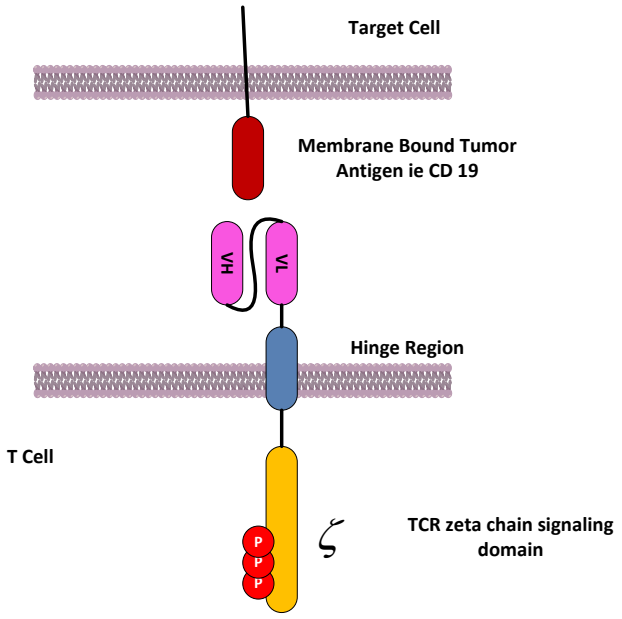
Evolution of CAR signaling capacities.

First generation CARs transmitted activating signals only via ITAM-bearing signaling chains like CD3ζ or FcεRIγ, licensing the engrafted T cells to eliminate tumor cells.

Second generation CARs contain an additional costimulatory domain (CM I), predominantly the CD28 domain. Signaling through these costimulatory domain leads to enhanced proliferation, cytokine secretion, and renders engrafted T cells resistant to immunosuppression and induction of AICD.

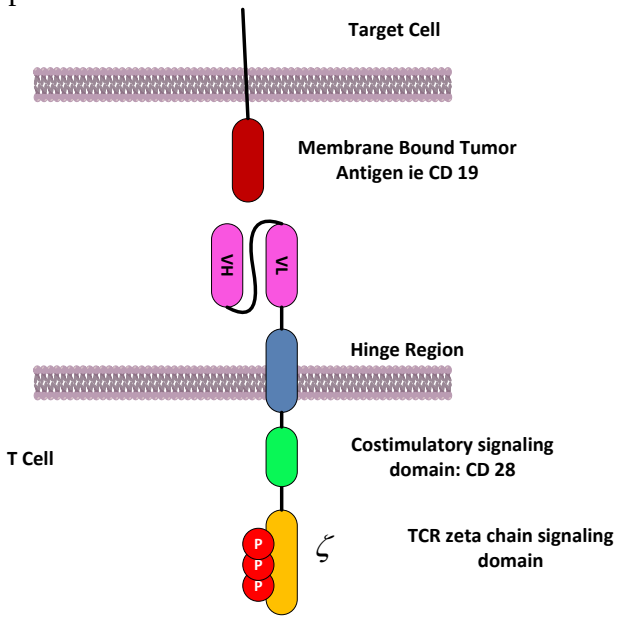
Third Generation CARs Recent developments fused the intracellular part of a second costimulatory molecule (CM II) in addition to CD28 and ITAM-bearing signaling chains, thus generating tripartite signaling CARs. T cells engrafted with third generation CARs seem to have superior qualities regarding effector functions and in vivo persistence.

The first generation shown below is the simplest.



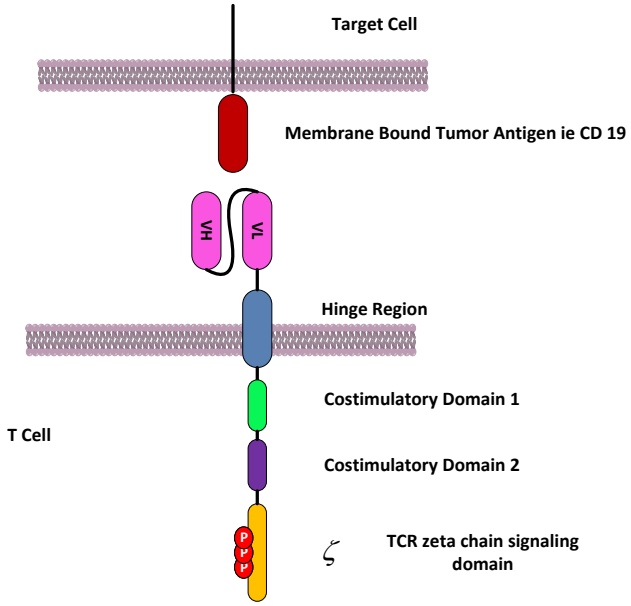
4.2.4 Second Generation

The second generation is as per below with the added element.



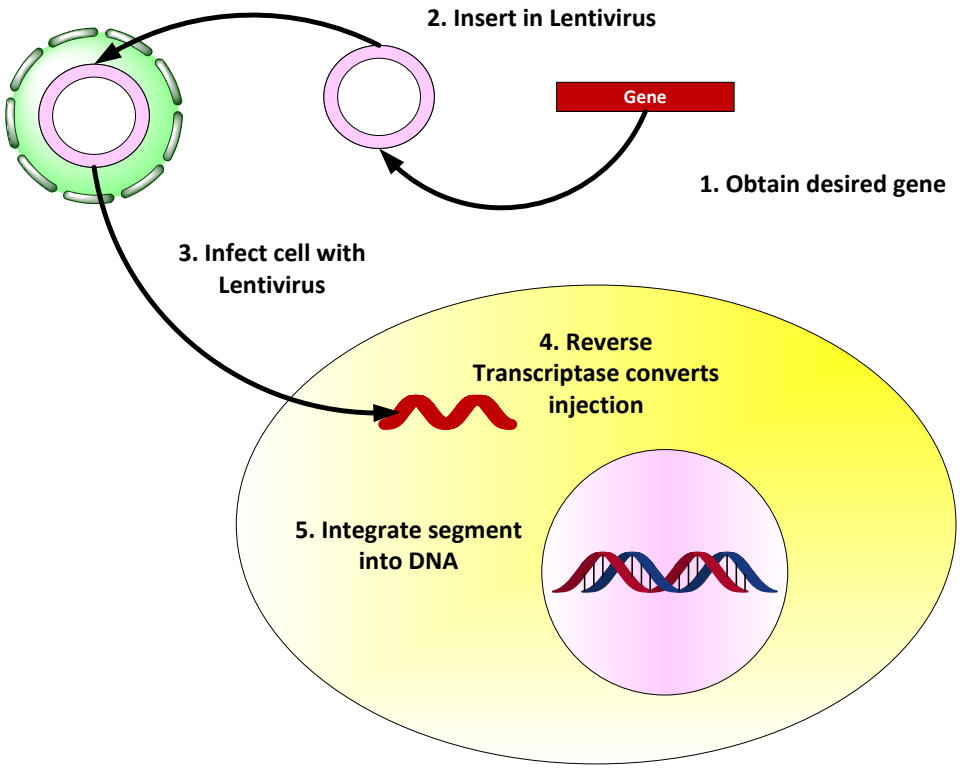
4.2.5 Third Generation

The third generation has added flexibility as shown below and described above.



4.2.6 Reverse Transcription and Gene Insertion

Now the insertion of the genes to create the previously described receptors uses a reverse transcription process. It is akin to what we see in HIV reverse transcription and specifically uses lentiviruses as the delivery mechanism.



As Naldini notes regarding lentiviruses:

Major hurdles for hematopoietic-stem-cell (HSC) gene therapy include achieving efficient ex vivo gene transfer into long-term repopulating HSCs, preventing activation of oncogenes by the nearby integration of a vector and controlling transgene expression to avoid ectopic or constitutive expression that leads to toxicity.

As compared to early generation gammaretroviral vectors (γ-RVs), HIV-derived lentiviral vectors result in more efficient gene transfer and stable, robust transgene expression in HSCs and their multilineage progeny. Extensive preclinical work indicated important features in vector biology and design that affect genotoxicity and highlighted strategies to alleviate it. The self-inactivating long terminal repeats (LTRs) and integration-site preferences of lentiviral vectors were shown to substantially alleviate insertional genotoxicity.

When tested in γ-RVs, the self-inactivating LTR design was shown to improve the safety of this platform as well. Retrospective analysis of several earlier trials suggests that disease background, transgene function, ex vivo culture and the efficiency of host repopulation can all influence the likelihood that insertional genotoxicity will manifest in a trial.

These data helped to shape the ideas that not all integrating vectors have the same effects and that genome-wide integration of improved vector designs, although still a mutagenic event, can be tolerated in the absence of aggravating circumstances. Self-inactivating lentiviral vectors are also being used to engineer T cells with chimeric antigen receptors (CARs) or T-cell antigen receptors for use in adoptive immunotherapy for the treatment of cancer. The advantages of this new platform in comparison to earlier-generation γ-RVs, which perform satisfactorily in this cell target, are yet to be fully established. Lentiviral vectors are thought to give rise to more robust and stable transgene expression in T cells in vivo, and could facilitate more efficient and versatile ex vivo gene transfer while supporting coordinated expression of multiple transgenes.

These advantages will become more relevant as the gene-therapy field implements refined strategies, such as improved T-cell manipulation to preserve T memory stem cells, or more demanding cell-engineering tasks, such as the co-expression of multiple CARs (to improve specificity) or a conditional safety switch/suicide gene (to improve safety).

4.2.7 CAR-T Cells and PSMA

Some work using CAR-T cells in PCa focusing on PSMA has been done. We begin with the work of Wang et al (2020) which notes:

Prostate-specific membrane antigen (PSMA) represents a suitable target for therapeutic purposes. Up to now, multiple ongoing clinical trials for prostate cancer CAR-T therapy based on PSMA-specific CARs have been reported.

One is a Phase I trial of prostate-specific membrane antigen (PSMA)-targeted CAR-T in CRPC patients (NCT01140373).

Another is a Phase I trial of PSMA-TGFβRDN CAR-T for CRPC (NCT03089203). The second trial is in purpose to evaluate the safety and feasibility of dual PSMA-specific/ TGFβ-resistant, CAR-modified autologous T cells (CARTPSMA-TGFβRDN cells) in CRPC patients.

The traditional CARs are generally composed of three sections, including extracellular antigen capturing section, transmembrane domain, and intracellular signal transduction part.

The extracellular antigen capturing section is usually served by single-chain fragment variable (scFv) or domain antibody with the size much smaller than ScFv, to specific recognize and capture the surface antigens in tumor cells; the transmembrane domain consists of the transmembrane region of CD3, CD8, CD28, or FcεRI which can fix antigen capturing proteins on the surface of T cells to transduce the signal into the cells via the binding or recognition of the tumor cells; while the intracellular signal transduction section is composed of CD8, CD28, or CD137 intracellular area and CD3ζ, which contains the immune-receptor tyrosine-based activation motif (ITAM).

Recently, more advanced generation of CAR-T was reported by introducing multiple costimulatory molecules or inducible costimulatory molecule, to further improve the tumor-killing abilities by enhancing T cell proliferation activity, cytotoxicity, and T cell survival rates.

Some CARs even contain additional proinflammatory factor and co-stimulatory molecule ligands (4-1 BBL and CD40L).

TGF-b has been proved to induce metastasis and neoangiogenesis. Expression of the dnTGF-bRII enhances antitumor immunity and T cell infiltration into tumors with potent antitumor responses. Results have been proved in the transgenic adenocarcinoma mouse prostate (TRAMP) mouse model of prostate cancer when utilizing this receptor. Recent results also showed that dominant-Negative TGF-b Receptor enhances PSMA-Targeted Human CAR T Cell Proliferation And Augments Prostate Cancer Eradication.

Interleukin 23 (IL-23), which is a heterodimeric cytokine composed of an IL12B (IL-12p40) subunit and the IL23A (IL- 23p19) subunit, is an inflammatory cytokine which plays a vital role in autoimmune diseases and in tumorigenesis.

Recent studies revealed that expression of the heterodimeric cytokine interleukin (IL)-23 is increased in human tumours, for IL-23 promotes inflammatory responses such as upregulation of the matrix metalloprotease MMP9, and increases angiogenesis but reduces CD8 T-cell infiltration [29]. IL-23 has also been proved of its tumor-promoting effect in mammary cancer mediated by infiltration of M2 macrophages and neutrophils in tumor microenvironment.

Recent study also showed that IL-23-induced immune cell activation aggravates gut inflammation and promotes growth of colon cancer. In prostate cancer study, IL-23 produced by myeloid-derived suppressor cells (MDSCs) and can activate the androgen receptor pathway in promoting cell survival and proliferation. Results also showed that antibody-mediated depletion of IL-23 restored sensitivity to androgen-deprivation therapy in mouse model.

All these studies highlighted the important role of IL-23 in tumor microenvironment. Based on these knowledges, we designed a panel of IL23mAb-PSMA-CARs, including PSMA-CAR, IL23mAb-T2A-PSMA-CAR, IL23mAb-PSMA-CAR, and PSMA-CAR (soluble IL23mAb). These CARs were designed by a novel IL-23 specific antibody with higher affinity, combined with previous PSMA specific monoclonal antibody.

And we found that IL-23mAb combined PSMA CARs worked better than PSMA CAR only in Prostate Cancer Eradication, and we further discussed the mechanisms among different IL-23mAb combined PSMA CARs in Prostate Cancer Eradication.

Montagner et al report other approaches as follows:

Prostate cancer (PCa) has become the most common cancer among males in Europe and the USA. Adoptive immunotherapy appears a promising strategy to control the advanced stages of the disease by specifically targeting the tumor, in particular through chimeric antigen receptor T (CAR-T) cell therapy. Despite the advancements of CAR-T technology in the treatment of hematological malignancies, solid tumors still represent a challenge. To overcome current limits, other cellular effectors than T lymphocytes are under study as possible candidates for CAR-engineered cancer immunotherapy. A novel approach involves the NK-92 cell line, which

mediates strong cytotoxic responses against a variety of tumor cells but has no effect on non-malignant healthy counterparts. Here, we report a novel therapeutic approach against PCa based on engineering of NK-92 cells with a CAR recognizing the human prostate-specific membrane antigen (PSMA), which is overexpressed in prostatic neoplastic cells.

More importantly, the potential utility of NK-92/CAR cells to treat PCa has not yet been explored. Upon CAR transduction, NK-92/CAR cells acquired high and specific lytic activity against PSMA-expressing prostate cancer cells in vitro, and also underwent degranulation and produced high levels of IFN-γ in response to antigen recognition.

Lethal irradiation of the effectors, a safety measure requested for the clinical application of retargeted NK-92 cells, fully abrogated replication but did not impact on phenotype and short-term functionality. PSMA-specific recognition and antitumor activity were retained in vivo, as adoptive transfer of irradiated NK-92/CAR cells in prostate cancer-bearing mice restrained tumor growth and improved survival. Anti-PSMA CAR-modified NK-92 cells represent a universal, off-the-shelf, renewable, and cost-effective product endowed with relevant potentialities as a therapeutic approach for PCa immunotherapy

They continue regarding the use of NK cells:

An increasing number of investigators believe that natural killer (NK) cells obtained from the peripheral blood of either the patient (autologous) or a healthy donor (allogeneic), might represent safer effectors for targeted cancer therapy than T cells. Additionally, the availability of continuously expanding NK cell lines provides a potentially unlimited source of effector cells, which can be investigated for genetic engineering but also hold the potential for the development as standardized off-the-shelf therapeutics for adoptive cancer immunotherapy (ACT). Among such NK cell lines, NK-92 cells are those that have been most thoroughly investigated and have already reached the testing phase in the clinical setting.

In this scenario, CAR-engineered NK-92 cells could offer a valid and cost-effective alternative to primary CAR NK or T cells, in particular in those cases where a suitable donor is not available or the sophisticated infrastructures needed for cell isolation, expansion, and genetic modification are lacking*.*

In this regard, the methodologies for continuous good manufacturing practice (GMP)-compliant expansion from an established master cell bank have been validated in the framework of early phase clinical trials with unmodified NK-92 cells, and can be easily adapted for large-scale production in centralized facilities.

This provides a further advantage that may be readily extended to CAR-engineered NK-92 variants. Indeed, in contrast to CAR approaches based on autologous or donor-derived primary cells, the genetic modification of NK-92 cells is not performed in a patient-individual setting under tight time constraints. Instead, a molecularly and functionally well-characterized cell product can be established that is endowed with a particular target specificity, and is continuously available independently from the time point of therapeutic application

They then conclude:

Overall, the results of this study highlight the potentialities of PSMA-specific CAR-modified NK-92 cells as a novel and exciting perspective for prostate cancer adoptive immunotherapy. Moreover, the potent antitumor activity, the immediate availability as a fully defined and characterized cell product, and the lack of obvious risks of manufacturing failures suggest that these cells can be advanced as a valid and cost-effective alternative to CAR-modified T cells. Finally, the robust ex vivo expansion of NK-92 cells to high numbers and their exquisite safety profile, as well as the ease of genetic modification, make this cell line an ideal platform for the development of off-the-shelf therapeutic CAR-engineered variants to target other solid tumors.

Minn et al have noted:

Chimeric antigen receptor (CAR) T cell therapy for hematologic malignancies is fraught with several unknowns, including number of functional T cells that engage target tumor, durability and subsequent expansion and contraction of that engagement, and whether toxicity can be managed.

Non-invasive, serial imaging of CAR T cell therapy using a reporter transgene can address those issues quantitatively. We have transduced anti CD19 CAR T cells with the prostate-specific membrane antigen (PSMA) because it is a human protein with restricted normal tissue expression and has an expanding array of positron emission tomography (PET) and therapeutic radioligands.

We demonstrate that CD19-tPSMA(N9del) CAR T cells can be tracked with DCFPyL PET in a Nalm6 model of acute lymphoblastic leukemia. Divergence between the number of CD19-tPSMA(N9del) CAR T cells in peripheral blood and bone marrow and those in tumor was evident. These findings underscore the need for non-invasive repeatable monitoring of CAR T cell disposition clinically…

There are limitations to the PSMA reporter approach for cell tracking. First, despite being a cell surface enzyme, PSMA is imaged using high-affinity inhibitors, as if it were a receptor.

The tPSMA(N9del) used here was developed specifically to eliminate the known cell internalization and turnover capability of PSMA to mitigate any potential effect on the biology of the CAR T cells. This places a theoretical limit on the sensitivity achievable, as PSMA will not concentrate the imaging.. agent within cells, i.e., amplify signal, as it is not an enzyme that turns over imaging substrate nor is it serving as a transporter in our instance. Nevertheless, we showed previously that it performed at least as well and even outperformed hNIS and the HSV1-sr39tk reporter systems when compared in vivo head to head. Second, because the PSMA-targeted imaging agents primarily undergo renal excretion, there is a strong signal in the kidneys unrelated to the presence of CAR T cells.

CAR T cells in or near the kidneys may be missed using the PSMA reporter. The clinical translation of the PSMA reporter system for CAR T cell imaging, particularly for CD19-expressing malignancies, would be relatively straightforward. The specificity of PSMA-targeted

imaging agents is very high (>90%), pitfalls of imaging with these agents have been published, and systems of reporting imaging findings have appeared, clearing a path for translation. As noted above, these agents are now offered worldwide and are proliferating. CAR T cell therapy is likewise seeing increasing use clinically and in an expanding number of indications.

Earlier work by Santoro (2014) appears to be the initial investigation using PSMA as a target. They had noted:

Prostate-specific membrane antigen (PSMA), best known as a prostate cancer–specific target, is a surface glycoprotein abundantly expressed on the endothelium of many solid tumors, but not on the normal vasculature). PSMA is a 750–amino acid type II membrane-bound protein transcribed from the PSMA locus, which encodes a number of splice variants, including multiple membrane-bound and cytosolic isoforms.

Interestingly, the ratio of membrane to cytosolic PSMA dramatically increases in prostate cancer. Recent studies have demonstrated that PSMA expression confers a proliferative advantage to tumor cells through its function as a hydrolase of poly- and gamma-glutamated folate. As such, it is presumed that PSMA plays a metabolic role on the activated tumor endothelium. Additional functions have also been ascribed to PSMA.

For example, mice lacking PSMA exhibit impaired angiogenesis as a result of defects in endothelial cell invasion. The expression of PSMA by the LNCaP prostate cancer cell line has been shown to induce the expression and secretion of IL6, which increases the proliferative potential of tumor cells.

Because the tumor endothelium has been shown to be an important source of IL6, it is conceivable that PSMA signaling is also involved in the production of IL6 from these cells.

Taken together, these data implicate PSMA as a contributor to tumor progression, and provide strong rationale for the generation of CAR T cells against the tumor endothelial cells on which it is expressed.

Here, we describe the development of CAR T-cell therapy directed against human (h)PSMA expressed by the tumor endothelium and provide proof of principle that this approach may be used to elicit tumor vascular disruption.

We demonstrate that anti-hPSMA CAR-bearing T cells function against endothelial targets in vitro regardless of the signaling domain incorporated into their design (z, 28z, BBz, or 28BBz). We also establish that the third-generation CAR T cells, containing the 28BBz signaling domain, are able to recognize primary tumor endothelial cells isolated from subjects with gynecologic cancer.

Furthermore, we show that in vivo the P28BBz T cells are able to resolve murine hemangioma and hemangiosarcoma tumors, which express hPSMA. Using state-of-the-art luciferase imaging technology, we show directly, for the first time, that CAR T cells are able to eliminate endothelial

cells within solid tumors and that vessel destruction results in secondary depletion of tumor cells, as well as reduced tumor burden.

Overall, our work demonstrates that PSMA is a valid target for CAR T-cell–mediated tumor blood vessel destruction, and provides insight into the importance of vascular disruption in the broader context of cancer therapeutics.

4.3 ANTI-CANCER VACCINES

Vaccines are potentially powerful tools in the treatment of many disorders. They prime the immune system to attack targeted cells and other antigen presenting elements. As part of the immunotherapy toolbox there is a growing facility in vaccines for targets cancers. In fact the recent rapid response to the corona virus pandemic could arguably be due to the extensive work on cancer related vaccines. As Handa et al note:

Despite Sipuleucel-T being a harbinger of hope, subsequent vaccines in PCa have demonstrated only a lukewarm clinical response. New vaccine strategies utilizing novel viral and bacterial vectors, as well as combinatorial approaches, are being tested to expand the ambit of immunotherapy by vaccines. However, several challenges exist in optimizing response to vaccination.

The immunosuppressive micro-environment of PCa is one such roadblock.

Addition of immunometabolic agents such as Indoximod, which relieves suppression of mammalian target of rapamycin (mTOR) and thus restores normal effector Tcell function or immune checkpoint inhibitors can potentially skew the balance towards an immunostimulatory tumor milieu. Radiotherapy has been proven to upregulate expression of TAAs and HLA as well as antigen processing molecules, and radiation-vaccination combination is being explored extensively in current clinical trials.

The role of androgen deprivation therapy in regulating immune response is well studied, is known to help in thymic regeneration, and can potentially render PCa cells immunesensitive. Enzalutamide in combination with vaccine has shown in vivo benefit in mouse models with advanced PCa, and, if proven, to be effective in humans may represent a paradigm shift in management of CRPC.

Another question is the selection of appropriate target antigen to induce a formidable and sustained immune response. Genetic modification resulting in amino acid substitutions in native TAAs to generate "mimotopes" can trigger Tcell cross-reactivity and generate a stronger immune response.

Targeting neoantigens produced by somatic mutations within the tumor can be another strategy, and has been trialed in malignancies with high mutational burden such as non-small cell lung cancer (NSCLC) and melanoma. Choosing an appropriate site and mode of delivery is an active research query as well.

Biomaterials to synthesize stable, nondegradable, precisely sized and shaped particles that are readily taken up by APCs are being developed. There is some evidence that intra-lymph node injection may induce a better immune response compared with subcutaneous delivery, and this requires further exploration in clinical trials.

Yet another approach can be using dendritic cell vaccines carrying mRNA derived from "cancer stem cells," which has shown clinical response in glioblastoma. While attempts are being made to develop more effective vaccines, several trials to unleash the full potential of vaccines in various combinations with other therapies are being conducted.

5 CRISPR APPROACHES

CRISPRS have become a significant tools in the modification of genes. Simply, CRISPRS are modifications of bacterial quasi-immune system mechanisms that target specific sections of DNA and then break that section apart allowing for the deletion, modification or insertion of a new DNA segment. The techniques have been modified and improved over the past decade. We examine this in the context of PSMA modifications and treatment of PCa.

5.1 CRISPR REDUX

We will now examine in more detail what a g is and how it functions. Let us begin by examining it in a bit more detail. As Randow et al state:

In archaea and bacteria, for example, even adaptive forms of resistance—long considered the hallmark of vertebrates—contribute to cell autonomous immunity, as exemplified by the clustered regularly interspaced short palindromic repeats (CRISPR) system, which recognizes foreign DNA in a sequence-specific manner. In metazoans, cellular self-defense synergizes with the whole-body protection provided by traditional immunity to confer pathogen resistance. Here, professional immune cells patrol their environment in search of pathogens, whereas cell-autonomous immunity guards both individual immune and non-immune cells against the immediate threat of infection.

Cellular self-defense thus has the potential to confer antimicrobial protection on most, if not all, cells….

In bacteria, foreign DNA is sensed and destroyed by the CRISPR system and restriction endonucleases. Because recognition motifs for most restriction endonucleases occur frequently in the host's own genome, these enzymes are paired with matching methyltransferases, which modify host DNA to demarcate it as "self." In eukaryotic cells, rather than being modified, DNA is largely sequestered inside the nucleus, which fosters the detection of foreign DNA in other compartments and allows the deployment of enzymes that mutate and/or degrade DNA without risk to the host genome.

Thus, as noted above, the original understanding was as a bacterial self-defense system. Now as Horvath and Barrangou state also concerning the original understanding:

Microbes have devised various strategies that allow them to survive exposure to foreign genetic elements. Although out-populated and preyed upon by abundant and ubiquitous viruses, microbes routinely survive, persist, and occasionally thrive in hostile and competitive environments.

The constant exposure to exogenous DNA via transduction, conjugation, and transformation have forced microbes to establish an array of defense mechanisms that allow the cell to recognize and distinguish incoming "foreign" DNA, from "self " DNA and to survive exposure to invasive elements. These systems maintain genetic integrity, yet occasionally allow exogenous

DNA uptake and conservation of genetic material advantageous for adaptation to the environment.

Certain strategies, such as prevention of adsorption, blocking of injection, and abortive infection, are effective against viruses; other defense systems specifically target invading nucleic acid, such as the restriction-modification system (R-M) and the use of sugar-nonspecific nucleases. Recently, an adaptive microbial immune system, clustered regularly interspaced short palindromic repeats (CRISPR) has been identified that provides acquired immunity against viruses and plasmids.

They also state:

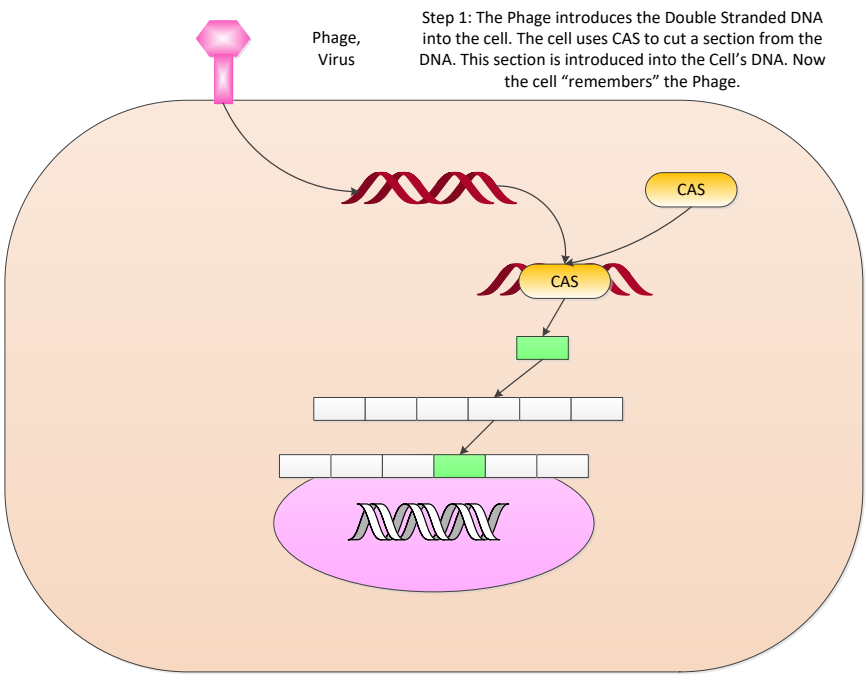
Microbes rely on diverse defense mechanisms that allow them to withstand viral predation and exposure to invading nucleic acid. In many Bacteria and most Archaea, clustered regularly interspaced short palindromic repeats (CRISPR) form peculiar genetic loci, which provide acquired immunity against viruses and plasmids by targeting nucleic acid in a sequence-specific manner.

These hypervariable loci take up genetic material from invasive elements and build up inheritable DNA-encoded immunity over time. Conversely, viruses have devised mutational escape strategies that allow them to circumvent the CRISPR/Cas system, albeit at a cost. CRISPR features may be exploited for typing purposes, epidemiological studies, host-virus ecological surveys, building specific immunity against undesirable genetic elements, and enhancing viral resistance in domesticated microbes.

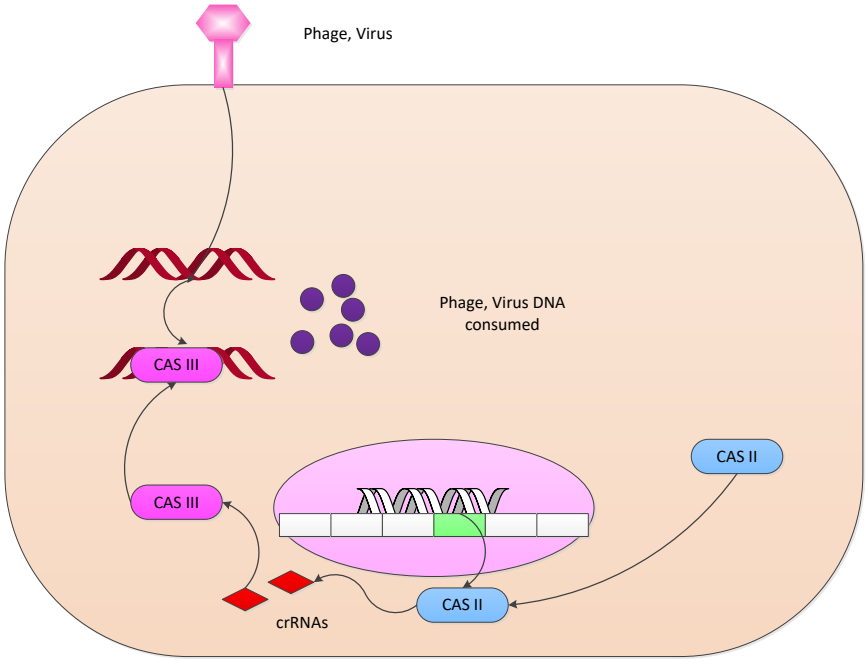
Thus, we first examine how CRISPR-Cas functions in its primal environment and then we take this to human environments where we can use it as an added tool in our genetic engineering toolkit.

5.1.1 CRISPR Dynamics

We now examine some of the dynamics of the CRISPR system. We start with the use of CRISPR in a bacterial cell. We assume the cell is attacked by some viral phage and the phage sends its RNA/DNA into the cell in anticipation of replication within the host. Now from Horvath and Barrangou (as modified) we have the following description for this initial portion of the process as shown below:



The Cas protein recognizes the invading DNA and transports a portion of it to the nuclear DNA and inserts it into the cell's DNA. How specifically Cas does this task is not yet well understood. The when another phage with the same or frankly similar DNA invades again, then Cas II is activated and the section of the DNA activates a Cas II which then consumes the invading DNA.



Now the above process is a natural part of the day-to-day activities of bacteria. But it also is a paradigm for deal with eukaryotic cells, namely cutting and pasting genes into cells.

5.1.2 Types of CRISPR

From Jinek et al, they discuss the three types of CRISPR systems:

There are three types of CRISPR/Cas systems.

The type I and III systems share some overarching features: specialized Cas endonucleases process the pre-crRNAs, and once mature, each crRNA assembles into a large multi-Cas protein complex capable of recognizing and cleaving nucleic acids complementary to the crRNA.

In contrast, type II systems process pre-crRNAs by a different mechanism in which a trans-activating crRNA (tracrRNA) complementary to the repeat sequences in pre-crRNA triggers processing by the double-stranded (ds) RNA specific ribonuclease RNase III in the presence of the Cas9 (formerly Csn1) protein. Cas9 is thought to be the sole protein responsible for crRNA-guided silencing of foreign DNA.

5.1.3 CRISPR Details

Current day biotechnology is in many ways a set of tools in a large tool box that handle the what and how of manipulating genes and their products. The tool and tool box metaphor are quite powerful and descriptive. The problem oftentimes is the why and also the integration of all of these elements from a technique to a technology.

In this brief paper we examine the CRISPR element less from that of a bench technique than as a technology that can be used in gene engineering. There is a mindset being explored that differs from that of the bench biologist. As an engineering approach one asks how can this technique be moved to a useful technology, and how deeply does one have to understand the underpinnings to use it effectively and safely.

One of the challenges of genetic engineering is the ability to select a specific gene and alter it, or add another gene or delete a gene. A key step in all of these is the ability to cut and paste at specific sites, at very specific sites. Now that one can read a gene in detail and when one knows what the desired result should be, then the cut and paste side is critical. Pasting is somewhat well known, especially if we have cut at the right location. CRISPR is a tool that does just that, it is a very accurate, fast, and low-cost gene cutting tool.

In this note we examine its structure from a systematic perspective. This will help understand what factors are the key factors and what elements should be understood. This is not a note for a bench biologist, it is not meant to be comprehensive. Yet unlike many of the simplified descriptions in the media I try to provide adequate depth with breath of applications.

We also try to establish the “gene engineering” tools that this mechanism can support. Finally, we discuss some of the concerns which have arisen in the use of CRISPRs.

To summarize, I refer to Mali et al who state:

Functioning of the type II CRISPR-Cas systems in bacteria.

Phase 1: in the immunization phase, the CRISPR system stores the molecular signature of a previous infection by integrating fragments of invading phage or plasmid DNA into the CRISPR locus as 'spacers'.

Phase 2: in the immunity phase, the bacterium uses this stored information to defend against invading pathogens by transcribing the locus and processing the resulting transcript to produce CRISPR RNAs (crRNAs) that guide effector nucleases to locate and cleave nucleic acids complementary to the spacer.

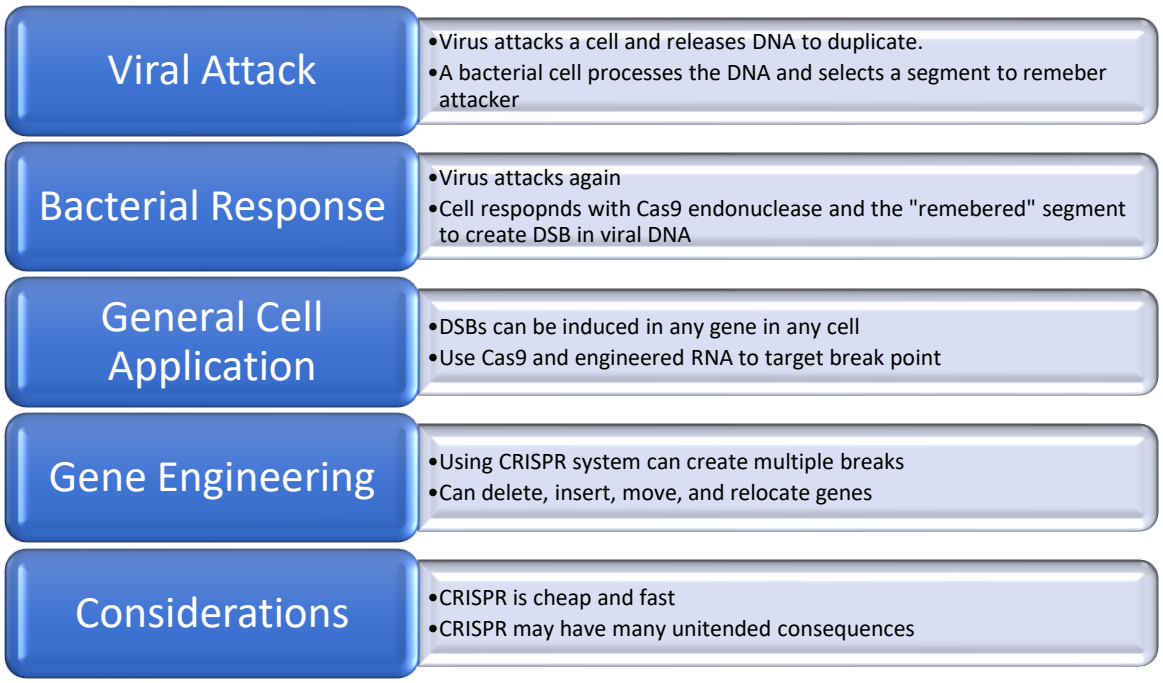
First, tracrRNAs hybridize to repeat regions of the pre-crRNA.

Second, endogenous RNase III cleaves the hybridized crRNAtracrRNA, and a second event removes the 5' end of the spacer, yielding mature crRNAs that remain associated with the tracrRNA and Cas9.

The complex cleaves complementary 'protospacer' sequences only if a PAM sequence is present.

Namely, this tool was seen developed in bacteria. The bacterium notes a section of the invading viral DNA, and then records that segment in its own DNA. Then when the virus attacks a second time, using the Cas9 nuclease protein produced by the bacteria then uses the RNA generated by the "remembered" sequences to attack the virus, and cut it so that it is made inoperable and it is digested.

Thus, in the report we follow the following considerations:



5.1.4 Bacterial Immunology

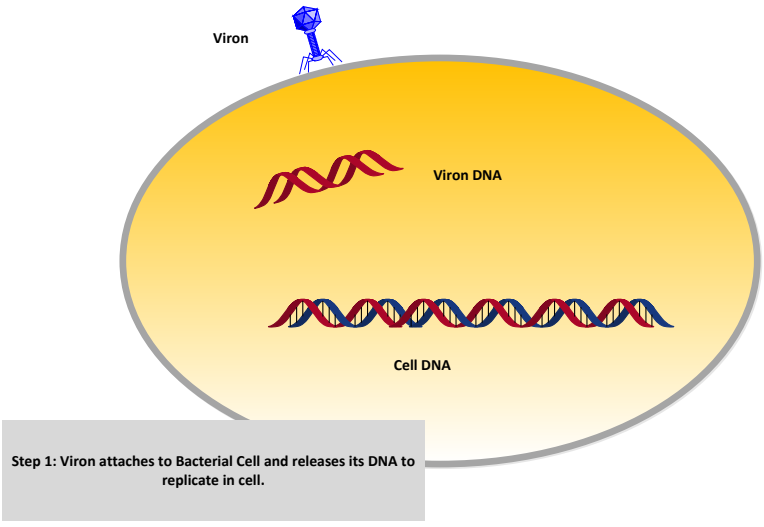
The CRISPR phenomenon comes from examining bacteria and their quasi-immune response to viral attacks. Simply stated;

Bacteria have developed a technique where they can recognize a foreign viral DNA segment and then "attack" is with an enzyme and a targeted RNA segment that results in the foreign DNA being broken and becoming ineffective. This bacterial process effectively kills the DNA of the invader, stops its reproduction and induces an autophagy.

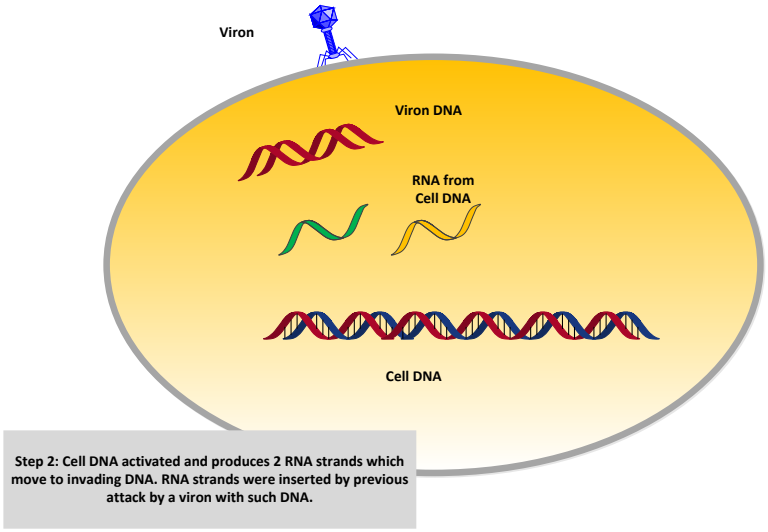
Now in discovering this process one then can take this same enzyme and modify the RNA that comes with it to match a location on some DNA we may be considering to manipulate and using this combo we can then cut DNA at a precise point anywhere we so desire. It is a powerful tool to cut DNA at a unique location. From there we can then add or delete DNA segments in a gene, in a somatic cell or in a germ line cell. It is fast and inexpensive and can be done in almost any lab.

Let us now begin with a virion attaching itself to a bacterium. We will assume that at some prior time some process has occurred where the bacteria had seen this for the first time. At this time that process is still a work in progress. But let us assume that this is a subsequent encounter and that in the process the bacteria has managed to record this prior encounter with a strand of DNA from that virion so that it can produce an RNA which is a map of some small segment of the virion's DNA.

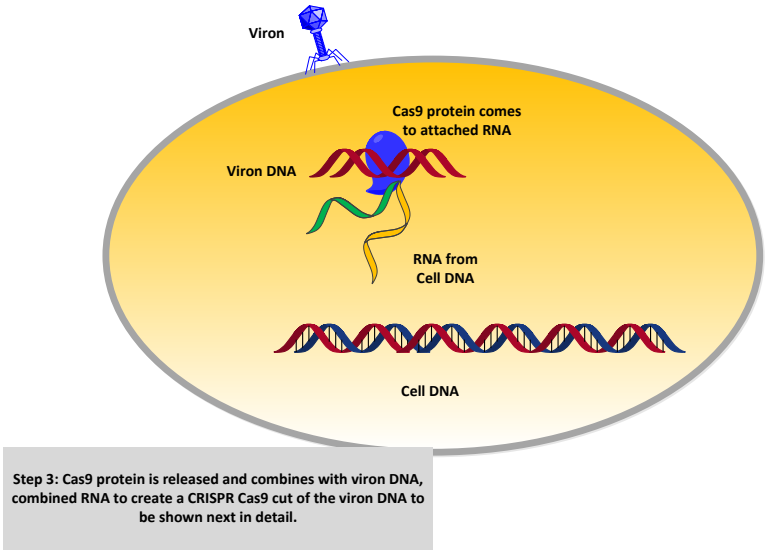
This is a lot of assumptions but it is generally where we start with the tool. We just want to know what the tool does not how it was made or even how in any detail it does what it does. In many ways we are looking at tools as a technician, namely use this tool this way and get this result. Leave the details for someone else.



Now when the virion gets into the cell there is produced RNA from the bacteria that was RNA based upon a prior encounter with this virion. Namely this RNA released matches a segment of DNA in the virion. Also remember that a virus just wants to use a cell, any cell, to reproduce itself, which frankly is just reproducing its DNA (or RNA). If the bacteria can use this knowledge of the attacker then what can it do to stop the reproduction, and potentially the organism's death.



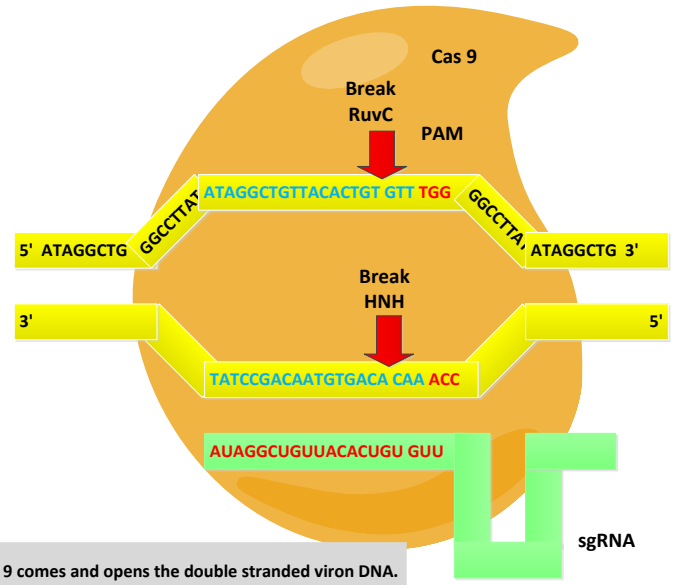
Now the RNA segments migrate to the virion DNA and along with a protein called cas9. The cas9 protein is the secret sauce of this tool.



The details of the operation are depicted in the graphic below. One must recall that this tool works but its operation is not fully understood. The Cas9 protein surrounds the desired site which has been selected by a combination of two factors. The first is the PAM sequence, in this case 3 nucleotides, nt, which act as a marker and then a 20 nt long matching strand down from the PAM. This key determines where the break occurs. In a bacteria's immune like response it needs

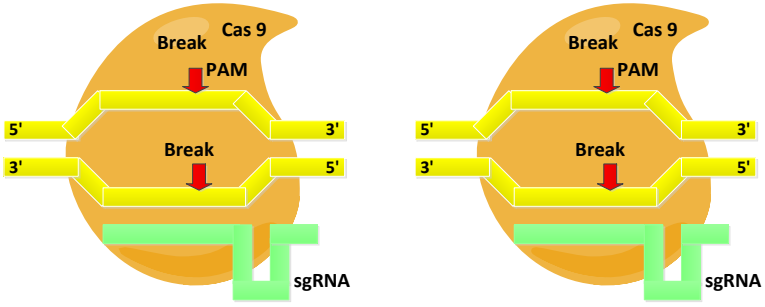
both, the PAM to be certain it does not kill itself and the 20 nt strand which gives a good marker for a specific site. In effect we have 23 nt for specific targeting. In genetic engineering cases we select the PAM as specified by the Cas9 source and then engineer the sgRNA element.

That yields a specific break site at 3 nt down from the end of the PAM. The two Cas9 fragments, RuvC and HNH are what cause the break.



Step 4: Cas 9 comes and opens the double stranded viron DNA. The sgRNA searches down the DNA looking for a match AND for the PAM sequence here being TGG or ACC. Then the sgRNA comes and binds to the bottom DNA. When binding complete two elements of Cas 9 break the DNA strands 3 nucleotides down from the PAM sequence. Then the viron DNA is defective and is dismembered by some form of autophagy. The viron DNA has been defeated.

The example below extends the above example to a double stranded break.



5.1.5 crRNA and tracrRNA

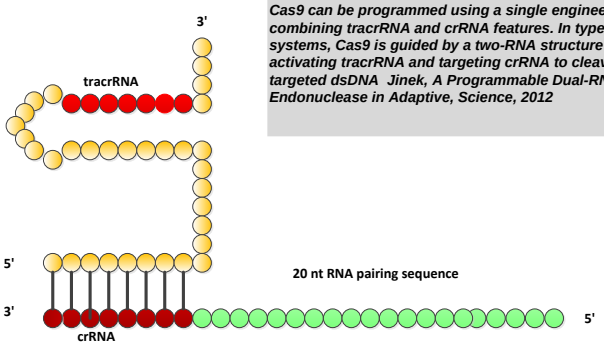
The two RNA segments, crRNA and tracrRNA can be configured in several ways. But they are the targets elements that are used to select where the break is to be. And once selected it is usually a double strand break. However single strand breaks can be accomplished as well.

As Jinek et al state:

In the expression and interference phases, transcription of the repeat-spacer element into precursor CRISPR RNA (pre-crRNA) molecules followed by enzymatic cleavage yields the short ***crRNAs that can pair with complementary protospacer sequences of invading viral or plasmid targets****. Target recognition by crRNAs directs the silencing of the foreign sequences by means of Cas proteins that function in complex with the crRNAs ….*

There are three types of CRISPR/Cas systems. The type I and III systems share some overarching features: specialized Cas endonucleases process the pre-crRNAs, and once mature, each crRNA assembles into a large multi-Cas protein complex capable of recognizing and cleaving nucleic acids complementary to the crRNA. In contrast, type II systems process precrRNAs by a different mechanism in which a ***trans-activating crRNA (tracrRNA)*** *complementary to the repeat sequences in pre-crRNA triggers processing by the double-stranded (ds) RNA specific ribonuclease RNase III in the presence of the Cas9 (formerly Csn1) protein. Cas9 is thought to be the sole protein responsible for crRNA-guided silencing of foreign DNA*

We demonstrate one variation of this below. Note the tracrRNA and its binding with crRNA and the 20 nucleotide ("nt") sequence which will select out the point at which we desire a break to be made.



Cas9 can be programmed using a single engineered RNA molecule combining tracrRNA and crRNA features. In type II CRISPR/Cas systems, Cas9 is guided by a two-RNA structure formed by activating tracrRNA and targeting crRNA to cleave site-specifically – targeted dsDNA Jinek, A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive, Science, 2012

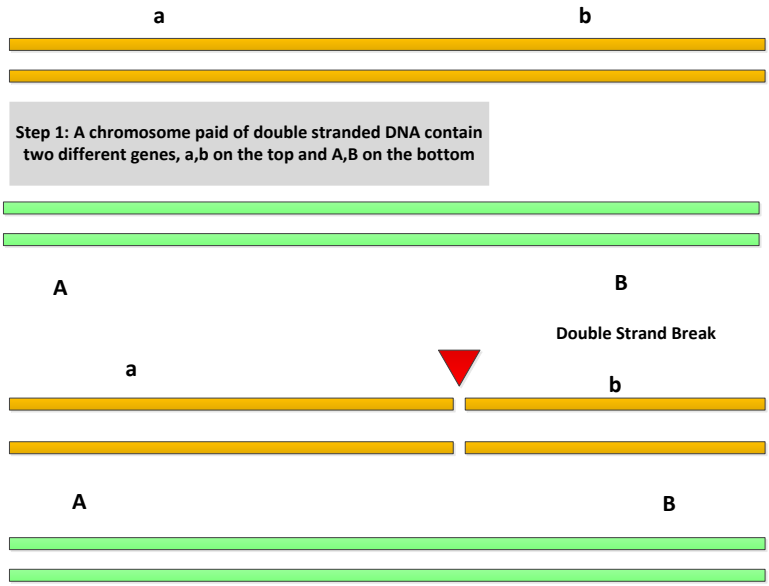
5.1.6 Gene Engineering

Now we ask; given a break at the right point what do we do next? That is the beginning of gene engineering. We briefly examine homologous repair, a somewhat well understood process, which uses the other chromosome as a template. The use of templates may also be done to insert new genes as well.

1. Let us start with a chromosome pair, one from each parent. We show this below.

2. Now we assume a double strand break, DSB, occurs on the top chromosome pair. We show this below:

3. Next we see a

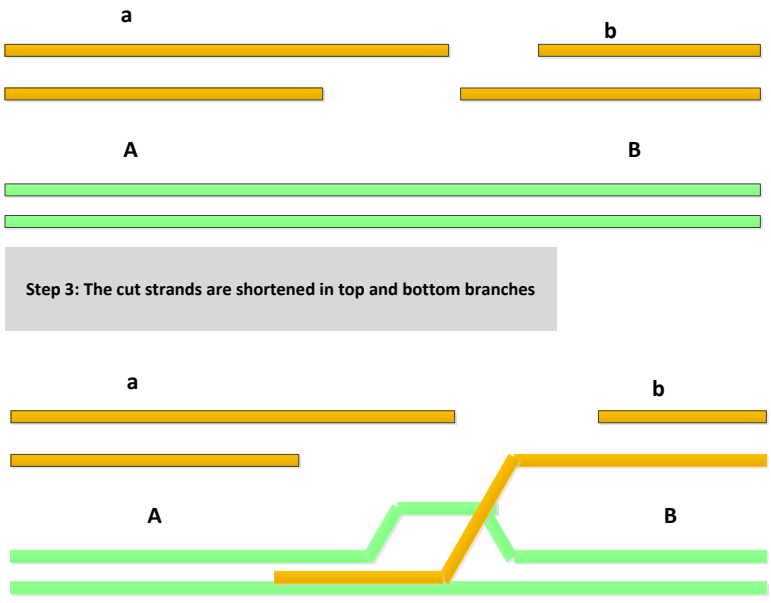


Step 1: A chromosome paid of double stranded DNA contain two different genes, a,b on the top and A,B on the bottom

Step 2: A double strand break occurs on the top DNA. For example we can select where this is with a CRISPR Cas9 approach just described

shortening of strands as shown below;

4. Then we see and elongation and use of the strand in the uncut DNA as a template. This can be used for other templates as insertion mechanisms.

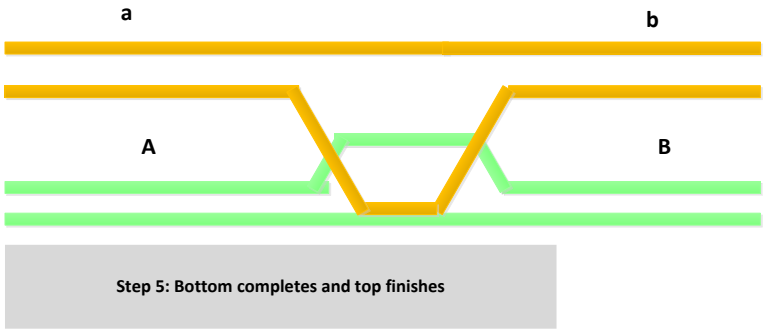


Step 3: The cut strands are shortened in top and bottom branches

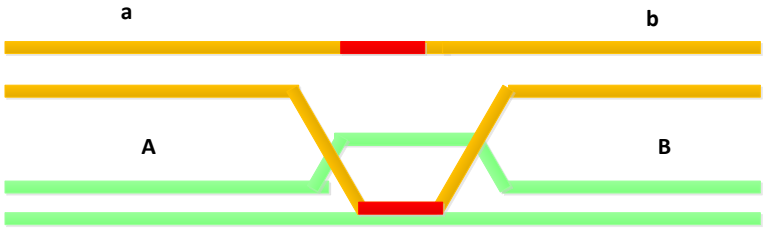
Step 4: The bottom branch uses the bottom branch as a renewal template reconstructing the full strand.

5. We see both top and bottom expanding and a crossover occurring.

6. We can see this also as an insertion mechanism.



Step 5: Bottom completes and top finishes



Step 6: A selected gene can be inserted and added, or it can equally well be removed by a two cut process.

Now this is a simple reconstruction of the process. Details are in Watson et al.

Actual gene editing with CRISPR can be complex. In the work by Komor et al they note:

Despite this breakthrough, genome editing still suffered from two major drawbacks. First, non-homologous end joining (NHEJ) also occurs at the site of DSBs, typically more efficiently than HDR, resulting in stochastic insertions and deletions (indels) of nucleotides at the target locus. While NHEJ-mediated genome editing is useful for gene disruption, indels are unwanted byproducts when precise genome editing is desired. Second, since the probability that a known meganuclease cleaves a particular target locus of interest is extremely small, either a meganuclease recognition site must be incorporated into the genomic locus of interest or a meganuclease must be engineered to cleave the target locus. Overcoming the first drawback is a focus of current research and will be discussed later in this Review.

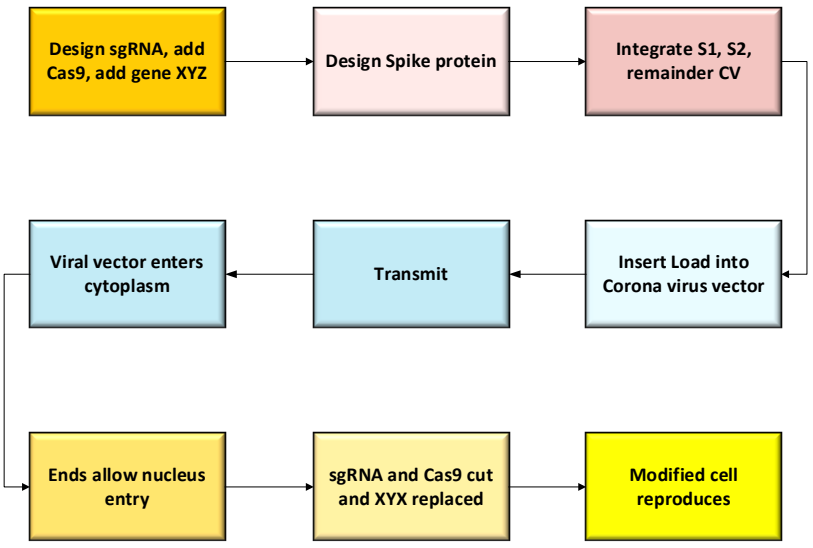
To address the second drawback, researchers turned to zinc-finger nucleases (ZFNs) and transcription-activator-like effector nucleases (TALENs), engineered nucleases based on arrays of naturally occurring DNA-binding domains fused to the nonspecific DNA cleavage domain from FokI. Because the amino acid sequences of zinc-finger arrays and TALE repeat arrays, unlike most DNA-binding proteins, can be readily designed to bind to virtually any target DNA sequence, ZFNs and TALENs can be engineered to cleave a target genomic loci with fairly high specificity. The design of ZFNs is complicated by their extensive proteinDNA contacts, however, and the cloning of TALEN genes is impeded by their highly repetitive nature. In addition, each new target locus requires the design, gene synthesis, expression, and validation of a new ZFN or TALEN. This significant barrier to genome editing—that each new target site requires the design and construction of a new nuclease—was substantially lowered by the advent of CRISPRCas9 as an RNA-guided DNA endonuclease.

In this system, a Cas endonuclease protein forms a complex with a ‘‘guide RNA’’ molecule and localizes to a target DNA sequence following simple guide RNA:genomic DNA base-pairing rules. The target DNA sequence (the protospacer) must be both complementary to the guide RNA and also contain a ‘‘protospacer-adjacent motif’’ (PAM), a short DNA sequence that is required for compatibility with the particular Cas protein being used).

While this new technology places a modest limitation on the number of genomic sites amenable to genome editing due to the PAM requirement, it replaces the complex protein design and engineering tasks associated with ZFNs and TALENs with the much-simpler task of designing a new guide RNA for each genomic site of interest using simple Watson-Crick base-.

The elucidation of the mechanics of CRISPR-Cas9, and its adaptation for use in eukaryotic genome, has had a transformative impact on the life sciences. The ease with which new DNA sequences can be targeted for genome editing has enabled scientists to rapidly discover new gene functions, develop new cell and animal models of diseases, and make substantial progress toward human therapeutics. In this Review, we summarize some of the recently developed tools that use CRISPR-Cas9 for the manipulation of mammalian genomes and their applications in basic science, biotechnology, and medicine.

The Zhang patent details some of the initial constructs. The process may appear as follows:



There are clearly a multiplicity of issues.

1. One must select the targeted gene appropriately.

2. The gene must be unique

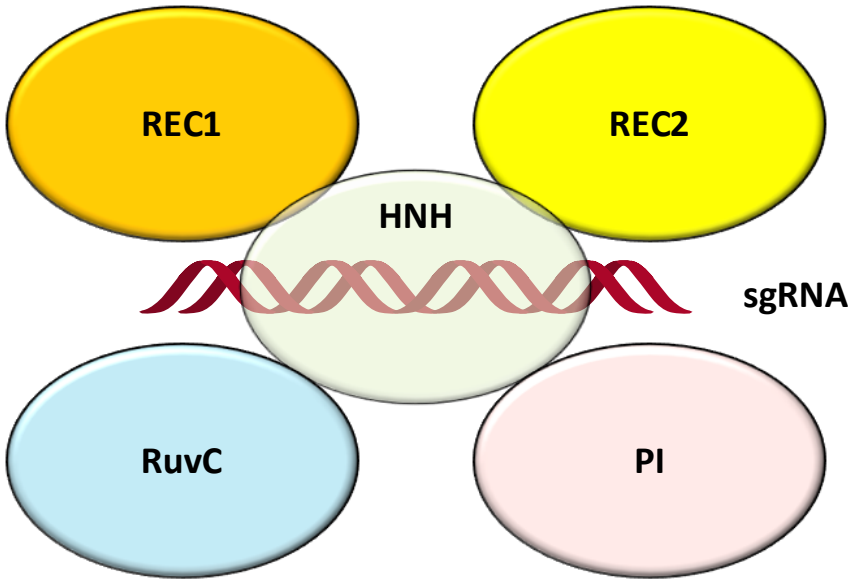
3. The combination of CRISPR and Cas9 must first enter the cell and then enter the nucleus

4. The combination must function in the nucleus properly

5. The loose ends must joint appropriately.

6. No other damage must be done.

The combination can be functionally viewed as follows:



Namely a set of intertwined proteins plus the RNA segments. These are large and thus can be a challenge to the entry to cytoplasm and then nucleus. We discuss this in Appendix 2.

5.2 CRISPR AND PSMA

As Thompson has recently noted:

Therapeutic gene manipulation has been at the forefront of popular scientific discussion and basic and clinical research for decades. Basic and clinical research applications of CRISPR–Cas9-based technologies and ongoing clinical trials in this area have demonstrated the potential of genome editing to cure human disease.

Evaluation of research and clinical trials in gene therapy reveals a concentration of activity in prostate cancer research and practice. Multiple aspects of prostate cancer care — including anatomical considerations that enable direct tumour injections and sampling, the availability of preclinical immune-competent models and the delineation of tumour-related antigens that might provide targets for an induced immune system — make gene therapy an appealing treatment option for this common malignancy. Vaccine-based therapies that induce an immune response and new technologies exploiting CRISPR–Cas9-assisted approaches, including chimeric antigen receptor (CAR) T cell therapies, are very promising and are currently under investigation both in the laboratory and in the clinic. Although laboratory and preclinical advances have, thus far,

not led to oncologically relevant outcomes in the clinic, future studies offer great promise for gene therapy to become established in prostate cancer care.

Prostate cancer lends itself to gene therapies, and multiple aspects of prostate cancer care make gene therapy an appealing treatment option for this common malignancy. Although laboratory and preclinical advances have not yet resulted in oncologically relevant outcomes in the clinic, future studies offer great promise for gene therapy to become established in prostate cancer treatment.

Prostate cancer gene therapy research and clinical trials established a foundation and path forwards for future advances in the field. Gene therapy strategies with potential applications in patients with prostate cancer include direct injection into a prostate tumour or systemic administration (vaccination).

Preclinical data on both strategies are promising, but large clinical trials have failed to demonstrate oncological benefit in patients. Further work, for example, modified vaccination strategies and the use of the CRISPR–Cas9 system, offers hope for future applications of gene therapy approaches in humans

As Marshall and Antonarakis have recently noticed:

Recently there has been an explosion of new agents being investigated for the treatment of prostate cancer. These modalities represent new therapies aimed at old targets, and new therapies addressing new targets.

This review will highlight three novel and emerging areas of treatment that have the potential to significantly impact the management of metastatic castration-resistant prostate cancer (mCRPC) in the near future: immunotherapy, poly ADP-ribose polymerase (PARP) inhibitors, and prostate-specific membrane antigen (PSMA)-targeted modalities.

Immunotherapy, particularly immune checkpoint blockers, PARP inhibitors, and PSMA-targeted therapies are all increasingly being studied for the treatment of mCRPC although none are currently FDA-approved specifically for prostate cancer. Together these three classes of treatments may drastically change the future landscape of mCRPC.

This review will cover what is currently known about the utility of these agents for the treatment of mCRPC, the areas of active research, and how these agents may be useful for patients in the future. It will also emphasize the notion of biomarker selection to help inform which patients are more likely to respond to these therapies.

Wei et al have examined CRISPR approaches to PCa as follows:

Androgens have been recognized to be primary causative agents of prostate cancer. Following binding to the androgen receptor (AR), androgens serve important roles in the carcinogenesis of prostate cancers. ARs serve an important role during all stages of prostate cancer, and inhibiting their function may help to slow prostate cancer growth. In the present study, the AR

gene was targeted in androgen-positive prostate cancer cells using the clustered regularly interspaced short palindromic repeats-associated protein (CRISPR/Cas) system. A total of three different single-guide RNAs (sgRNAs) were designed according to the three different target sites in the AR gene.

The optimal sgRNA with a specific target effect was effectively screened to cleave the AR gene in androgen-positive prostate cancer cell lines, and to suppress the growth of androgen-sensitive prostate cancer in vitro. The AR-sgRNA-guided CRISPR/Cas system was able to disrupt the AR at specific sites and inhibit the growth of androgen-sensitive prostate cancer cells; further studies demonstrated that the decreased cell proliferation was due to cellular apoptosis. The results of the present study suggested that the CRISPR/Cas system may be a useful therapeutic strategy for the treatment of prostate cancer.

Zhen et al have similarly noted:

The potent ability of CRISPR/Cas9 system to inhibit the expression of targeted gene is being exploited as a new class of therapeutics for a variety of diseases. However, the efficient and safe delivery of CRISPR/Cas9 into specific cell populations is still the principal challenge in the clinical development of CRISPR/Cas9 therapeutics. In this study, a flexible aptamer-liposome-CRISPR/Cas9 chimera was designed to combine efficient delivery and increased flexibility.

Our chimera incorporated an RNA aptamer that specifically binds prostate cancer cells expressing the prostate specific membrane antigen as a ligand. Cationic liposomes were linked to aptamers by the post-insertion method and were used to deliver therapeutic CRISPR/Cas9 that target the survival gene, polo-like kinase 1, in tumor cells. We demonstrate that the aptamer-liposome-CRISPR/Cas9 chimeras had a significant cell-type binding specificity and a remarkable gene silencing effect in vitro.

Furthermore, silencing promoted a conspicuous regression of prostate cancer in vivo. Importantly, the approach described here provides a universal means of cell type–specific CRISPR/ Cas9 delivery, which is a critical goal for the widespread therapeutic applicability of CRISPR/Cas9 or other nucleic acid drugs.

6 OBSERVATIONS

We now consider some observations regarding the issues discussed herein. Namely when examining targetable ligand or ligand like surface antigens, we can consider therapeutic options as a combination of multiple techniques, not just the classic single path targeting. We know in general that many of the new and innovative approaches have responses often at best 30-40%. Namely the majority of patients have no response. We also know that the same effect occurred early in various chemotherapies. Thus we argue for multiple therapeutic approaches using many of the tools we discuss herein. This is therefore not a discussion of PSMA in vacuo but a vehicle to consider the amalgam of options against many targets.

6.1 PCA HAS SIGNIFICANT GENOMIC DIVERSITY AND DOES THIS COMPLEXITY MAKE THERAPEUTIC EXCESSIVELY DIFFICULT?

There is a multiplicity of gene mutations in PCa. As Armenia et al note:

Comprehensive genomic characterization of prostate cancer has identified recurrent alterations in genes involved in androgen signaling, DNA repair, and PI3K signaling, among others. However, larger and uniform genomic analysis may identify additional recurrently mutated genes at lower frequencies. Here we aggregate and uniformly analyze exome sequencing data from 1,013 prostate cancers. We identify and validate a new class of E26 transformation-specific (ETS)- fusion-negative tumors defined by mutations in epigenetic regulators, as well as alterations in pathways not previously implicated in prostate cancer, such as the spliceosome pathway.

We find that the incidence of ***significantly mutated genes (SMGs)*** *follows a long-tail distribution, with many genes mutated in less than 3% of cases. We identify a total of 97 SMGs, including 70 not previously implicated in prostate cancer, such as the ubiquitin ligase CUL3 and the transcription factor SPEN. Finally, comparing primary and metastatic prostate cancer identifies a set of genomic markers that may inform risk stratification.*

Recently Han et al have examined the genomic complexity and they conclude:

In case of transcriptome-based molecular subtyping of breast cancer, expression of a set of 494 breast tumor-cell–intrinsic genes was defined to overcome heterogeneity arising from the stroma.

Following this strategy, we used previously deposited prostate-tissue epithelial cell- lineage–specific gene expression profiles assessed by bulk and single-cell RNA sequencing.

We discovered four transcriptomic subtypes of primary prostate adenocarcinoma – luminal A, luminal S, AVPC-I and AVPC-M. Our classification partly overlapped with earlier findings from multi-omics-based or marker-based clustering approaches. However, subtype definitions were not absolute, resulting in classification of ~25% of tumors as mixed. Strikingly, KLK3 and ACP3 mRNA expression levels, encoding PSA and PAP, respectively, showed potential to identify

subtypes; this was further supported by serum PSA and PAP levels measured before prostatectomy or docetaxel chemotherapy.

Identification of cancer molecular subtypes has deepened our understanding of cancer biology and clinical implications, including therapeutic target identification. In breast cancer docetaxel adjuvant chemotherapy was not beneficial in the luminal A population or in patients with ER-positive and HER2-negative cancers.

We claim that such findings can be translated to PCa. Our analysis suggests that luminal A subtype, with the strongest AR activity, should undergo treatment with the new AR target agents and avoid taxanes if diagnosed in advanced stage.

Encouragingly, data from recent molecular profiling of mCSPCs support that the SPOP-mutated tumors are less likely to become castration-resistant. In contrast, TP53 inactivation, a distinguishing feature of AVPC subtypes from the luminal tumors, was predictive of abiraterone and enzalutamide outcomes in mCRPCs. For the AVPC-M subtype which predicted to be resistant to both docetaxel and AR signaling inhibitors, DNA damage-inducing agents (purine analogues) might be tried. Indeed, clinical trials showed that AVPC-ms (+) tumors can benefit from DNA-damaging platinum-based chemotherapies in addition to cabazitaxel….

Both KLK3 (coding PSA) and ACP3 (coding PAP) genes are regulated by AR. An integrative analysis on their gene promoters and enhancers public ChIP-seq data that ACP3 gene is regulated also by ETS family transcription factors (SPI1, ERG) and inflammation-related factors (STAT1, STAT3). This might be the underlying mechanism that PSA/PAP ratio in general decreases following androgen deprivation therapy.

In other words, PSA/PAP ratio may reflect the activity of alternative signaling pathways (ETS family, STATs) that leads to early onset castration-resistance.

In addition to the fact that SPOP-driven prostate tumors (Luminal A) and ERG-driven tumors (Luminal S and AVPCs) exist in mutually exclusive manner, our analysis support that the AVPCs mostly arise in ERG-driven tumors by losses of PTEN and p53. ERG activation coordinate with PTEN loss in prostate cancer progression, and it is likely that loss of p53 on top of ERG/PTEN loss promote androgen-independent tumor growth and metastasis. These are shared characteristic of AVPC-I and AVPC-M, and imply that the AVPCs harbor significant chromosomal instability that potentially be associated with microtubule stabilizer's anti-tumor mechanism. We further speculate that compared to AVPC-I, AVPC-M have less vascularization, slow in cell cycle, and frequent genetic mutations of PI3K-Akt-mTOR pathway that promotes resistance to taxanes.

Our finding does not contradict earlier reports that ERG induces taxane resistance in CRPC.

Rather, it underscores the importance of radiographic and clinical responses over PSA response in mCRPC cases, where increasing numbers of PSA-low neuroendocrine–like cancers are seen.

We argue that ETS-fusion tumors can be subdivided - luminal S, AVPC-I and AVPC-M, and the taxane-resistance and castration-resistance might be dependent on molecular contexts such as combined losses of PTEN, TP53 or PIK3CA associated with ETS fusion

6.2 PSMA INITIATION AND CONTROL APPEARS NOT WELL DOCUMENTED. IT POSSIBLY PRESENTS AN ALTERNATIVE THERAPEUTIC.

The key question is: what drives the overproduction of PSMA? The upstream pathway that initiates the overproduction is uncertain. We believe that this is a critical next step. In addition with other similar markers we need the same result.

6.3 THERE ARE A MULTIPLICITY OF OTHER TARGETS LIKE PSMA. ARE SOME BETTER? CAN ONE USE POLYSPECIFIC AB TO ATTACK MULTIPLE SUCH LIGANDS SIMULTANEOUSLY?

Many researchers have examined multiple other targets, We present just a few:

6.3.1 P2X4

Maynard et al have reported on a target as follows:

P2X4 belongs to the P2 purinergic receptor family that is commonly upregulated in cancer and is associated with poorer outcomes.

Herein, we report that the P2X4 purinergic receptor is overexpressed in PCa, associated with PCa metastasis, and a driver of tumor development in vivo. We observed P2X4 protein expression primarily in epithelial cells of the prostate, a subset of CD66+ neutrophils, and most CD68+ macrophages. Our analysis of tissue microarrays representing 491 PCa cases demonstrated significantly elevated P2X4 expression in cancer compared to benign tissue spots, in prostatic intraepithelial neoplasia, in cancer from White compared to Black men, and in PCa with ERG positivity or with PTEN loss.

High P2X4 expression in benign tissues was likewise associated with the development of metastasis after radical prostatectomy. Treatment with P2X4-specific agonist CTP increased transwell migration and invasion of PC3, DU145, and CWR22Rv1 PCa cells. P2X4 antagonist 5-BDBD treatment resulted in a dose-dependent decrease in viability of PC3, DU145, LNCaP, CWR22Rv1, TRAMP-C2, Myc-CaP, BMPC1, and BMPC2 cells and decreased DU145 cell migration and invasion. Knockdown of P2X4 attenuated growth, migration, and invasion of PCa cells. Finally, knockdown of P2X4 in Myc-CaP cells resulted in significantly attenuated subcutaneous allograft growth in FVB/NJ mice. Collectively, these data strongly support a role for the P2X4 purinergic receptor in PCa aggressiveness and identifies P2X4 as a candidate for therapeutic targeting.

6.3.2 TENB2

Boswell et al have reported on TENB2:

TENB2, a transmembrane proteoglycan protein, is a promising target for antibody drug conjugate (ADC) therapy due to overexpression in human prostate tumors and rapid internalization.

We previously characterized how predosing with parental antiTENB2 monoclonal antibody (mAb) at 1 mg/kg in a patient-derived LuCap77 explant model with high (3+) TENB2 expression could (i) block target-mediated intestinal uptake of tracer (< 0.1 mg/kg) levels of radiolabeled anti-TENB2-monomethyl auristatin E ADC while preserving tumor uptake, and (ii) maintain efficacy relative to ADC alone.

Here, we systematically revisit this strategy to evaluate the effects of predosing on tumor uptake and efficacy in LuCap96.1, a low TENB2-expressing (1+) patient-derived model that is more responsive to ADC therapy than LuCap77. Importantly, rather than using tracer (< 0.1 mg/kg) levels, radiolabeled ADC tumor uptake was assessed at 1 mg/kg – one of the doses evaluated in the tumor growth inhibition study – in an effort to bridge tissue distribution (PK) with efficacy (PD).

Predosing with mAb up to 1 mg/kg had no effect on efficacy. These findings warrant further investigations to determine whether predosing prior to ADC therapy might improve therapeutic index by preventing ADC disposition and possible toxicological liabilities in antigen-expressing healthy tissues.

6.3.3 STEAP1

Danila et al have reported on a transmembrane protein STEAP1:

Six-transmembrane epithelial antigen of the prostate 1 (STEAP1) is highly expressed in prostate cancers.

DSTP3086S is a humanized immunoglobulin G1 anti-STEAP1 monoclonal antibody linked to the potent antimitotic agent monomethyl auristatin E. This study evaluated the safety and activity of DSTP3086S in patients with metastatic castration-resistant prostate cancer…. Six-transmembrane epithelial antigen of the prostate 1 (STEAP1) is a multitransmembrane protein believed to act as an ion channel or transporter protein.

As a cell surface protein frequently expressed in prostate cancer, with limited expression in nonprostate tissues, STEAP1 is an ideal candidate for antibody-derived therapies in patients with mCRPC.

DSTP3086S is an antibody-drug conjugate (ADC) that contains the humanized immunoglobulin G1 antiSTEAP1 monoclonal antibody MSTP2109A linked through a protease labile linker, maleimidocaproylvaline-citrulline p-aminobenzyloxycarbonyl, to a potent antimitotic agent, monomethyl auristatin E (MMAE). …

In summary, DSTP3086S demonstrated an acceptable safety profile, with evidence of antitumor activity confirming that the targeting of STEAP1-expressing mCRPC tumors with

an ADC is feasible. Although DSTP3086S would require optimization for further clinical development, these data may inform development of novel ADCs, chimeric antigen receptor T cells, and immune cell–recruiting bispecific antibodies that target STEAP1.

This appears to be another attractive target. If we were to generate a polyAb with targets of STEAP1 and PSMA then one suspects the specificity would be exceptionally high. It is through mechanisms such as these that we believe both therapeutic delivery and immunotherpeutics can be successfully achieved. However we still face the TME issues which we believe can dominate mets in PCa.

7 APPENDIX 1: Pleckstrin Homology

The Pleckstrin homology is a domain or proteins that is the 11th most common such domain in the human proteome. The domain can bind a multiplicity of other proteins and is a facilitator of intracellular signalling[19]. (see Lemmon) We have seen in the above discussions the impact of PH and thus it is useful to delineate it is more detail/

There has been some work, see DeSemir et al. on the targeting of the Pleckstrin Homology, "PH", as an additional target for controlling multiple cancers. As DeSemir et al state regarding the Pleckstrin Homology Domain-Interacting Protein (PHIP) (slightly edited):

Given the important role of Akt in the IGF (Insulin Growth Factor) axis, we then assessed whether Phip was involved in Akt activation. …

Because of the uncharacterized role of PHIP in cancer, we performed cDNA microarray analysis to identify the global patterns of gene expression after suppression of Phip expression. Significance analysis of microarrays identified 51 down-regulated genes (including Igf2 and Tln1) and 184 overexpressed genes … Thus, PHIP can regulate the expression of upstream mediators of the IGF axis and downstream mediators of tumor cell invasion.

Having demonstrated Phip's functional role in promoting murine melanoma metastasis, we examined its impact on human melanoma progression.

We performed immunohistochemical analysis of PHIP expression on a tissue microarray cohort of 345 patients with primary cutaneous melanoma …

High levels of PHIP expression were found in each histological subtype of melanoma and accounted for almost one-third of the melanomas in this cohort.

High PHIP expression correlated significantly with the presence of ulceration, an adverse prognostic factor incorporated into the staging classification for melanoma whose biologic basis is poorly understood…

PHIP overexpression was significantly predictive of reduced distant metastasis-free survival … and disease-specific survival …

PHIP overexpression was an independent predictor of DMFS and DSS…

PHIP overexpression directly correlated with the progression of distant metastases, and with reduced survival, in both murine and human melanoma.

The human PHIP gene resides on the 6q14.1 locus. Deletions of the 6q arm have been shown in melanoma and have been suggested as a possible diagnostic marker. …

[19] Recall that "homology" means a shared ancestry. Pleckstrin is a protein found in platelets. https://meshb.nlm.nih.gov/record/ui?name=pleckstrin Thus the shared ancestry.

FISH analysis revealed that the PHIP locus was still present in all 78 melanomas examined.

Importantly, there was a significant correlation between PHIP copy number (assessed as a percentage of cells with three or more copies) and the corresponding PHIP immunohistochemical scores …

Melanomas with immunohistochemical scores of 1–3 had a significantly higher percentage of cells with increased copy number compared with melanomas with a PHIP score of 0 .. In addition, 80.6% of PHIP 3 melanomas had three or more copies of the PHIP locus.

Although we found no evidence of amplification, because PHIP copy number remains comparable with chromosome 6 centromeric copy number increased copy number of the PHIP melanomas for β-catenin mutations at six different sites (previously described in melanoma; COSMIC database) and found no mutations at any of these sites.

These results show that PHIP levels can be activated in a unique molecular subset of melanoma independent of mutations in these other four genes.

This brief summary of the work makes PHIP an interesting and attractive target. It presents a pathway element which is more a facilitator rather than a major participant (see Weinberg). As we shall note later from DeSemir et al, they contend that the PHIP target presents a more universal target especially for those melanomas which do not have well defined mutations in BRAF, NRAS or PTEN. As we have discussed previously, for example, PTEN mutations, loss of control in the Akt pathway, is often an end game in cancer progression, for example in prostate cancer and many others.

We will attempt to assemble some of the literature and present a brief summary of this area. In many ways it is distinct from the pathway targets themselves since the PH targets are smaller and often are found in many of the pathway elements. The PHD. Pleckstrin Homology Domain, has received significant interest by other researchers especially regarding its pathway control effects. For example Hirano et al have examined it in CML and Miyamoto et al in cardiology and the Akt pathway.

We first examine Pleckstrin then its homology and its function. We begin first with Pleckstrin. Pleckstrin is a specific protein which is found in blood platelets. The name is derived using the concatenation of the phrases: **P**latelet and **LE**ukocyte **C K**inase substrate and the KSTR string of amino acids. It is located on 2p13.3.

7.1 DESCRIPTION

Now the Pleckstrin Homology is defined as:

Pleckstrin homology domain (PH domain) is a protein domain which consists of approximately 120 amino acids. The PH domain is present in various proteins which are key elements of intracellular signaling as well as constituents of the cytoskeleton.

This domain can bind phosphatidylinositol lipids within biological membranes (such as phosphatidylinositol (3,4,5)-trisphosphate and phosphatidylinositol (4,5)-bisphosphate. PIP3 and PIP2), and proteins such as the βγ-subunits of heterotrimeric G proteins, and protein kinase C.

Through these interactions, PH domains play a role in recruiting proteins to different membranes, thus targeting them to appropriate cellular compartments or enabling them to interact with other components of the signal transduction pathways.

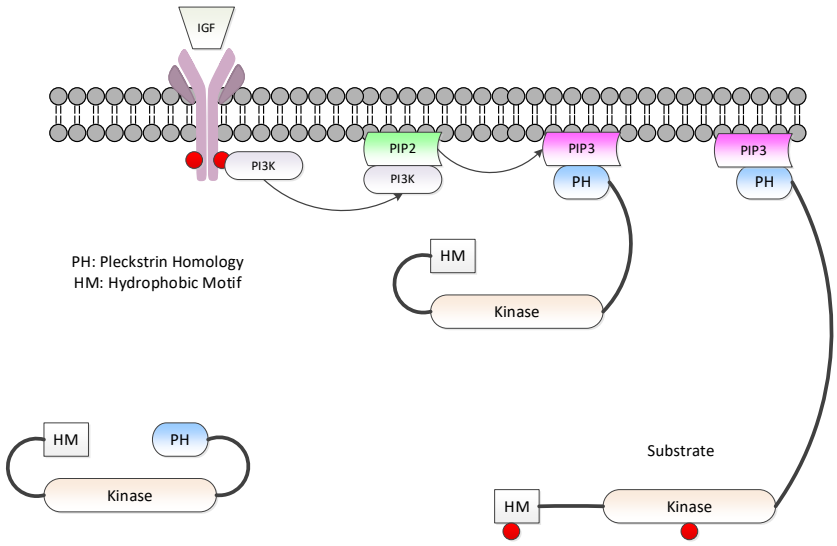
PH domains can be found in many different proteins, such as ARF. Recruitment to the Golgi in this case is dependent on both PtdIns and ARF. A large number of PH domains have poor affinity for phosphoinositides and are hypothesized to function as protein binding domains. Proteins reported to contain PH domains belong to the following families:

i. Pleckstrin, the protein where this domain was first detected, is the major substrate of protein kinase C in platelets. Pleckstrin is one of the rare proteins to contain two PH domains.
ii. Ser/Thr protein kinases such as the Akt/Rac family, the beta-adrenergic receptor kinases, the mu isoform of PKC and the trypanosomal NrkA family.
iii. Tyrosine protein kinases belonging to the Btk/Itk/Tec subfamily.
iv. Insulin Receptor Substrate 1 (IRS-1).
v. Regulators of small G-proteins like guanine nucleotide releasing factor GNRP (Ras-GRF) (which contains 2 PH domains), guanine nucleotide exchange proteins like vav, dbl, SoS and *S. cerevisiae* CDC24, GTPase activating proteins like rasGAP and BEM2/IPL2, and the human break point cluster protein bcr.
vi. Mammalian phosphatidylinositol-specific phospholipase C (PI-PLC) isoforms gamma

Discussion of PH in cancer is somewhat sparse and limited in detail. Bunz has a short reference (p 191) and Weinberg also has passing comments in several locations, and Schulz on p. 120.

7.2 PH AND PATHWAYS

The following is from Marks et al and shows how the PH domain can act as a binding and activating substrate in the overall pathway cascade process. It can unwrap from the complex protein of which it is a part, and then it can attach to a membrane protein and this allows activation, in the case below, by phosphorylating the resulting domain substrate. This simple model offers also a mechanism to block pathway activation as well.



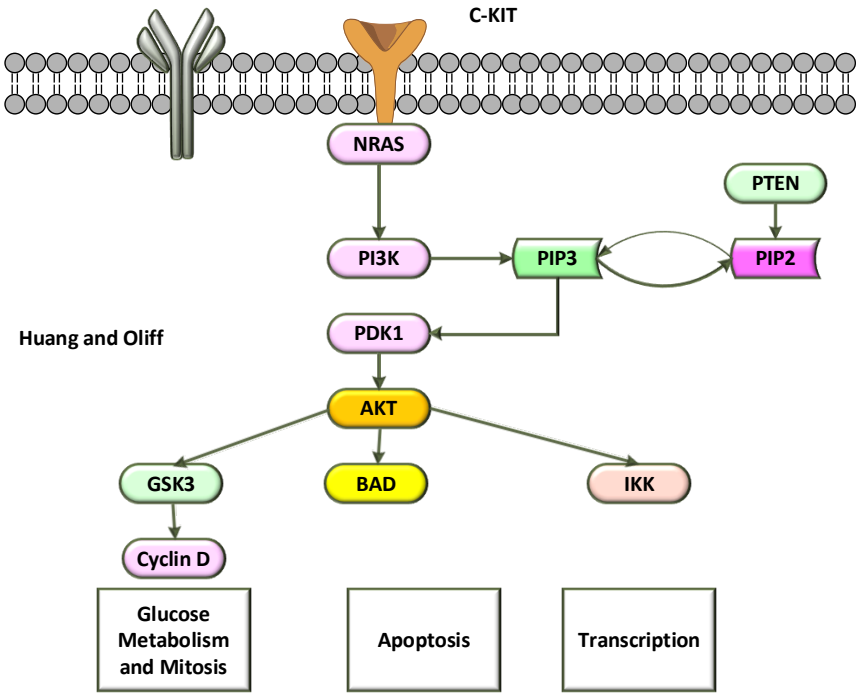
From: Marks et al, p 178.

As Huang and Oliff state regarding the PH domain:

There are three members of the AKT (PKB) family. They are widely expressed and implicated in apoptosis, insulin signalling and growth regulation. All three contain a pleckstrin lipid-binding domain (PH Domain) and are activated at the membrane by upstream kinases. Candidates for this upstream regulatory activity include integrin-linked kinase, PDK-1, and possibly AKT itself. In addition, AKT activity is regulated indirectly through modulation of lipid metabolism.

The loss of PTEN (a protein and lipid phosphatase) activity and the gain of PI3K (a protein and lipid kinase) activity correlate with AKT activity and binding of AKT to the membrane lipid, PI(3)P. The PI3K inhibitor wortmannin has already been shown to inhibit AKT signalling. Some proteins that have been shown to be substrates of AKT and relevant to apoptosis are listed. Antagonists of AKT kinase activity should inhibit signalling through these downstream effectors.

We demonstrate this pathway selectivity and control below. Here we have modified a Figure from Huang and Oliff to make the point that loss of PTEN control or over-activation of the Akt pathway can result in excess of proliferation and suppression of apoptosis. This is generalized below:



PTEN is a major control protein in pathway management. As Chow and Baker had stated in an earlier description of the effects of PTEN:

Soon after the discovery of its PIP3 phosphatase activity, PTEN was found to negatively regulate the PI3K/AKT pathway . Generation of PIP3 by growth factor-stimulated PI3K activity results in membrane recruitment of the serine–threonine kinase AKT via its pleckstrin homology (PH) domain, and activation by phosphoinositide-dependent kinases (PDK1 and 2) . Numerous AKT substrates have been identified affecting a broad range of cellular activities .

A few that have been implicated in oncogenic transformation include the Forkhead family of transcription factors (FOXO), p27^{KIP1}, MDM2, GSK3, BAD, IKK-b, and tuberin (TSC2), a negative regulator of mTOR. The specific targets phosphorylated by AKT vary with physiological stimuli and cell context and the mechanism for this selection is unclear. The complexity of this pathway is further underscored by the recent finding that mTOR can act both upstream and downstream of AKT activation. The raptor–mTOR complex can phosphorylate and activate AKT while the raptor–mTOR complex, which regulates growth and protein translation, can be activated downstream of AKT .

PTEN-mediated regulation of the PI3K/AKT pathway results in cell context-dependent effects on cell size, proliferation and survival. A dominant-negative form of AKT rescues the lethality caused by PTEN deficiency in flies. This strongly suggests that AKT is the major critical downstream target of PTEN activity ..

The impact of Akt has been understood now for quite a while and the BRAF facilitation when mutated has become a focal element of the control mechanism. However PH also plays a significant role and this too has been understood. As Dehaia states:

PI3-kinase triggers signaling through multiple pathways, many of which are thought to associate with cell growth and survival. PTEN, working in opposition to PI3-kinase, is therefore associated with cell death or arrest signals. Phospholipid residues such as PtdIns(3,4,5)P_3 are present in cells upon stimulation by several growth factors, such as platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), and epidermal growth factor (EGF).

Upon activation by growth factor, proteins containing a pleckstrin-homology (PH) domain are recruited to the membrane 3 where they associate with phospholipids. One of the PH domain-containing proteins relevant in this pathway is the serine-threonine kinase, AKT, also known as PKB or RAC1*.*

AKT, in turn, and as a consequence of lipid binding, alters its conformation to allow two of its residues, threonine 308 and serine 473, to be phosphorylated and therefore become active.

The kinase responsible for phosphorylation of threonine 308 is phosphonositide-dependent kinase 1 (PDK1), an enzyme which also contains a PH domain and is therefore dependent on lipid binding for its full activity. There is some preliminary evidence, predominantly from in vitro studies, that a second lipid-dependent, PH domain-containing enzyme, ILK (integrin-linked kinase), is responsible for phosphorylation of the serine 473.

Further, a recent paper has proposed that the kinase responsible for Ser 473 phosphorylation might in fact be PDK1, when it associates with certain specific proteins, such as PDK1 interacting fragment (PIF), as seen by in vitro studies. By dephosphorylating D3 residues on PtdIns$_3$ and PtdIns(3,4)P_2, PTEN works in opposition to the PI3K/AKT pathway and therefore counteracts cell survival mechanisms elicited by this signaling. The mechanisms of cell survival associated with AKT appear to involve multiple pathways, including growth factors, cytokines, c-myc overexpression, UV irradiation, and matrix detachment.

One of the known signals activated by AKT is its phosphorylation of the Bcl-2 family member, BAD: phosphorylation of BAD results in suppression of apoptosis. AKT has also been reported to counteract the apoptotic response of several cellular factors. Recently, the transcription factor NF-kappaB has been implicated in the apoptotic response antagonized by the PI3K/AKT pathway

Thus we have demonstrated that PH activateable proteins such as Akt can be deactivated if it were possible to focus on the PH Domain as a target sector. Recent work has demonstrated that in some detail.

7.3 CURRENT UNDERSTANDING

We now will examine some of the current understanding of PH and its implications in melanoma specifically. We examine the work of two other groups and then readdress that of DeSemir et al.

As Farang Fallah et al state:

As a major substrate of the insulin receptor, insulin receptor substrate 1 (IRS-1) plays a central role in transducing insulin-dependent signals that regulate biological processes such as cell growth and cellular uptake of glucose. IRS-1 is a modular protein comprised of an N-terminal region harboring a pleckstrin homology (PH) domain, followed by a phosphotyrosine binding (PTB) domain that cooperatively ensures selective recognition and efficient substrate phosphorylation by the activated insulin receptor (IR). The C-terminal portion contains multiple tyrosine phosphorylation motifs which serve as docking sites for the recruitment of various SH2 (Src-homology 2) domain containing signaling molecules, such as phosphatidylinositol 3-kinase (PI 3-kinase), Grb-2 adaptor protein, and SHP2 (SH2 containing phosphatase 2) tyrosine phosphatase, which in turn elicit the activation of biochemical cascades that promote the metabolic and growth responses to insulin….

In the present study we demonstrate that overexpression of either PHIP or IRS-1 alone in muscle cells was not sufficient in promoting transport of GLUT4 to plasma membrane surfaces This is consistent with other observations, indicating that activation of IRS-1-associated signaling effectors such as PI 3-kinase, although necessary, is not sufficient for GLUT4 activation.

Notably, growth factors such as platelet-derived growth factor and interleukin-4 can activate PI 3-kinase as efficiently as insulin and yet fail to stimulate glucose transport in insulinsensitive cells.

One possible explanation is that additional PHIP/IRS-1/PI 3-kinase-independent pathways are required to coordinate GLUT4 intracellular routing. Indeed, recent evidence points to a novel insulin-responsive pathway that recruits flotillin/CAP/CBL complexes to IR-associated lipid rafts in the plasma membrane, an event which is thought to potentiate GLUT4 docking to the cell surface after IR activation.

Our data, however, provide support for the involvement of PHIP/IRS-1 complexes in glucose transporter GLUT4 translocation in muscle cells. Specifically, the use of DN-PHIP or IRS-1 PH domain constructs known to interfere with efficient IR–IRS-1 protein interaction, and hence productive signal transduction from IRS-1 to PI 3-kinase, blocked the ability of insulin to stimulate GLUT4 mobilization in L6 myoblasts and inhibited insulin-stimulated actin cytoskeletal reorganization, a process required for the productive incorporation of GLUT4 vesicles at the cell surface. Moreover, this inhibition did not coincide with changes in the autophosphorylation status of the IR.

As Barnett et al state:

Akt/PKB (protein kinase B) is a serine/threonine kinase which has a key role in the regulation of survival and proliferation. There are three isoforms of human Akt (Akt1, Akt2 and Akt3) and they all have an N-terminal PH (pleckstrin homology) domain and a kinase domain separated by a 39-amino-acid hinge region. The PH domains have approx. 60% identity and the kinase domains are >85% identical.

The hinge region is the least conserved at approx. 28% identity. The Akt active-site residues, described in a recent report on the crystal structure of Akt2 containing an ATP analogue and a

peptide substrate , are the same in all three iso-enzymes. Based on the high degree of homology between the AGC protein kinase family members, the identification of specific active-site inhibitors has been predicted to be difficult. The identification of Akt iso-enzyme-specific inhibitors seemed to be an even greater challenge….

Two Akt inhibitors were identified that exhibited isoenzyme specificity. The first compound (Akt-I-1) inhibited only Akt1 while the second compound (Akt-I-1,2) inhibited both Akt1 and Akt2 with IC_{50} values of 2.7 and 21 μM respectively. Neither compound inhibited Akt3 nor mutants lacking the PH (pleckstrin homology) domain at concentrations up to 250 μM.

These compounds were reversible inhibitors, and exhibited a linear mixed-type inhibition against ATP and peptide substrate. In addition to inhibiting kinase activity of individual Akt isoforms, both inhibitors blocked the phosphorylation and activation of the corresponding Akt isoforms by PDK1 (phosphoinositide-dependent kinase 1).

A model is proposed in which these inhibitors bind to a site formed only in the presence of the PH domain. Binding of the inhibitor is postulated to promote the formation of an inactive conformation. In support of this model, antibodies to the Akt PH domain or hinge region blocked the inhibition of Akt by Akt-I-1 and Akt-I-1,2. These inhibitors were found to be cell-active and to block phosphorylation of Akt at Thr_{308} and Ser_{473}, reduce the levels of active Akt in cells, block the phosphorylation of known Akt substrates and promote TRAIL (tumour-necrosis-factor-related apoptosis inducing ligand)-induced apoptosis in LNCap prostate cancer cells.

We can now return to the results of DeSemir et al. As they look to the usefulness of PHIP they state:

Although melanomas with mutant v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) can now be effectively targeted, there is no molecular target for most melanomas expressing wildtype BRAF. Here, we show that the activation of Pleckstrin homology domain-interacting protein (PHIP), promotes melanoma metastasis, can be used to classify a subset of primary melanomas, and is a prognostic biomarker for melanoma.

Systemic, plasmid based shRNA targeting of Phip inhibited the metastatic progression of melanoma, whereas stable suppression of Phip in melanoma cell lines suppressed metastatic potential and prolonged the survival of tumor-bearing mice.

The human PHIP gene resides on 6q14.1, and although 6q loss has been observed in melanoma, the PHIP locus was preserved in melanoma cell lines and patient samples, and its overexpression was an independent adverse predictor of survival in melanoma patients. In addition, a high proportion of PHIP-overexpressing melanomas harbored increased PHIP copy number.

PHIP-overexpressing melanomas include tumors with wild-type BRAF, neuroblastoma RAS viral (v-ras) oncogene homolog, and phosphatase and tensin homolog, demonstrating PHIP activation in triple-negative melanoma. These results describe previously unreported roles for PHIP in

predicting and promoting melanoma metastasis, and in the molecular classification of melanoma.

This demonstrates the extended ability of PHIP to enhance the usefulness of other markers. They continue as follows:

As a result, "triple-negative melanoma" patients, whose tumors harbor wild-type v-Raf murine sarcoma viral oncogene homolog B1 (BRAF), neuroblastoma RAS viral (vras) oncogene homolog (NRAS), and phosphatase and tensin homolog (PTEN) (the most common mutations observed in melanoma), are not candidates for most targeted therapies developed to date.

This as we have noted before is one of the most significant findings. We know that BRAF mutations are currently targeted with some beneficial albeit temporally limited results. Perhaps PHIP may add an additional targeting.

They conclude:

Overexpression or mutation of genes that play important roles in tumor progression. A high proportion of melanomas are characterized by BRAF, NRAS, or PTEN mutations. However, the molecular basis of triple-negative melanomas lacking these mutations is poorly characterized. Our results suggest that PHIP levels may be used to classify some melanomas that lack these three mutations. It is likely that additional molecular aberrations will be identified to further characterize triple-negative melanomas.

Along with recent studies demonstrating that the IGF axis is activated in melanomas with acquired resistance to BRAF inhibition, these studies have identified IGF signaling as an important alternative pathway to promote melanoma progression. Overall, our studies identify PHIP as a molecular mediator of melanoma progression that also appears to function in the setting of a subset of triple-negative melanomas.

Clearly BRAF, NRAS and PTEN mutations are well defined targets, BRAF especially for melanoma and PTEN seems to span a wide number of cancers. However if they are not changed the PHIP mutation seems more in line with wit an reasonable target.

8 APPENDIX 2: CYTOPLASM AND NUCLEUS TRANSPORT

There is a challenge in getting various therapeutics from outside the cell to the cytoplasm and then into the nucleus. We avoid the nucleolus for the time being. We consider first the CRISPR approach.

8.1 OPTIONS

As Shalaby et al have noted:

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) genome editing system has been the focus of intense research in the last decade due to its superior ability to desirably target and edit DNA sequences. The applicability of the CRISPR-Cas system to in vivo genome editing has acquired substantial credit for a future in vivo gene-based therapeutic. Challenges such as targeting the wrong tissue, undesirable genetic mutations, or immunogenic responses, need to be tackled before CRISPR-Cas systems can be translated for clinical use.

Hence, there is an evident gap in the field for a strategy to enhance the specificity of delivery of CRISPR-Cas gene editing systems for in vivo applications. Current approaches using viral vectors do not address these main challenges and, therefore, strategies to develop non-viral delivery systems are being explored. Peptide-based systems represent an attractive approach to developing gene-based therapeutics due to their specificity of targeting, scale-up potential, lack of an immunogenic response and resistance to proteolysis.

In this review, we discuss the most recent efforts towards novel non-viral delivery systems, focusing on strategies and mechanisms of peptide-based delivery systems, that can specifically deliver CRISPR components to different cell types for therapeutic and research purposes.

Moreover they discuss the limitations which are critical:

The key challenges of translating CRISPR therapeutics to the clinic center around off-target effects, where unwanted genetic modifications occur, and the lack of efficient delivery systems that can specifically target CRISPR to the tissue of interest while avoiding undesirable gene editing in the remaining parts of the body. These limitations are addressed in current research by enhancing CRISPR's gene recognition precision, and by developing and testing novel delivery vectors capable of tissue targeting

They then discuss the delivery mechanisms:

CRISPR-Cas nuclease, and gRNA can be delivered in several formulations:

1-DNA

i. Two plasmids; one encoding the protein and one encoding the gRNA.
ii. A single plasmid encoding both components.

2- RNA

i. Cas protein encoded in a messenger RNA (mRNA) and the gRNA as an in vitro transcribed synthetic oligonucleotide

3- Protein

i. Cas protein and a synthetic gRNA oligonucleotide.
ii. Cas protein and gRNA RNP complex.

They conclude:

Although the field of CRISPR therapy has flourished during the last decade, there remains a need for developing appropriate targeted delivery systems to advance its use to the clinic. Developing non-viral delivery systems for CRISPR have been a subject of immense research to avoid the safety concerns associated with viral vectors. Thus far, most targeted delivery systems employed for CRISPR-Cas are dependent on ligand-decorated liposomes or nanoparticles, which require complex design or may be toxic to achieve cell-specific receptor recognition. Peptide-based non-viral delivery systems contain properties that offer advantages such as chemical diversity, low toxicity, resistance to proteolysis and ability for specific targeting. The non-covalent complexing of CRISPR-Cas components with peptides through charge–charge interactions represents a one-step, simple, safe, translatable, and customizable method for specific tissue targeting. Targeting CRISPR-Cas systems to the cells of interest carries a great deal of hope for biomedical research and for achieving the safety levels required for clinical translation. More research is needed to test the efficacy of peptide-based systems for the targeted in vivo delivery of CRISPR-Cas to different tissues.

They continue:

Different delivery platforms of Clustered Regularly Interspaced Short Pallindromic Repeats (CRISPR)-Cas system in human cells.

Possible forms of CRISPR-Cas cargo to be delivered to cells (DNA, RNA, or protein) and intracellular processing:

(1) a CRISPR-Cas encoding plasmid is transcribed to CRISPR-associated (Cas) messenger RNA (mRNA) and a guide RNA (gRNA) encoding plasmid is transcribed into a single-guide RNA (sgRNA).

(2) mRNA moves to the cytoplasm to be translated into a Cas nuclease,

(3) a sgRNA/Cas ribonucleoprotein complex (RNP) is imported into the nucleus.

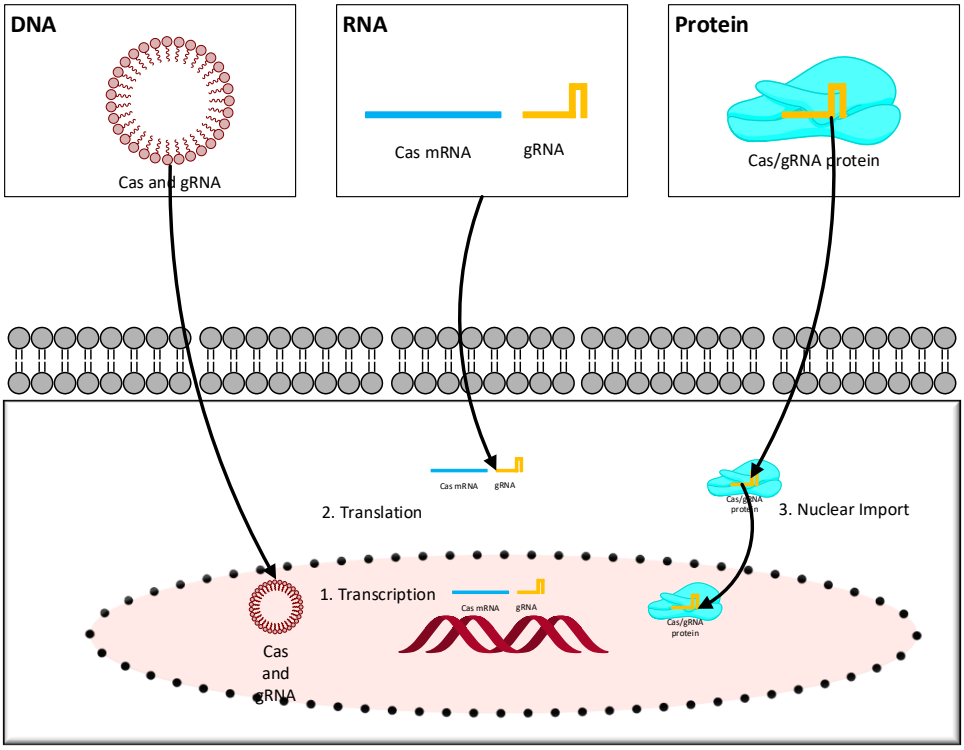
(4) gRNA/Cas RNP performs gene editing at the target site.

From the delivery point of view, using a single component is more efficient and practical because it does not require the design and co-loading of a separate delivery vector for each component. Moreover, gene editing cannot occur without the presence of both components in the same cell (i.e., the chance for a cell to receive two components is less than receiving one component).

For therapeutic purposes, using plasmids may have potential safety concerns because introduced DNA may interfere with the host genome causing insertional mutagenesis. A general consensus is that it is preferable to deliver the Cas nuclease as a functional protein because it saves the time during which the plasmid needs to be expressed; naked gRNA is relatively unstable and may get diffused or degraded, becoming unavailable to form a RNP complex with the nuclease when protein expression is completed.

Ultimately, the goal of each strategy is the localization of a RNP complex inside the nucleus of host cells, and delivering it as a functional RNP complex is considered the most straightforward and efficient strategy, requiring the least amount of intracellular processing. The RNP complex also significantly protects the delicate gRNA molecule from degradation, and it has a short half-life which reduces the chances for off-target mutations to occur.

We graphically depict these below:



8.2 Nuclear Localization Signals

A critical step in the process is getting into the nucleus. We know there are many ways to enter the cell cytoplasm. Getting further into the nucleus often uses nuclear localization signals or

sequences. These are amino acid sequences that are at the beginning and end of the sequence which is to be entered.

As Maggio et al note:

Enhancing the intracellular delivery and performance of RNA-guided CRISPR-Cas9 nucleases (RGNs) remains in demand. ***Here, we show that nuclear translocation of commonly used Streptococcus pyogenes Cas9 (SpCas9) proteins is suboptimal.***

Hence, we generated eCas9.4NLS by endowing the high-specificity eSpCas9(1.1) nuclease (eCas9.2NLS) with additional nuclear localization signals (NLSs). We demonstrate that eCas9.4NLS coupled to prototypic or optimized guide RNAs achieves efficient targeted DNA cleavage and probe the performance of SpCas9 proteins with different NLS compositions at target sequences embedded in heterochromatin versus euchromatin. Moreover, after adenoviral vector (AdV)-mediated transfer of SpCas9 expression units, unbiased quantitative immunofluorescence microscopy revealed 2.3-fold higher eCas9.4NLS nuclear enrichment levels than those observed for high-specificity eCas9.2NLS. This improved nuclear translocation yielded in turn robust gene editing after nonhomologous end joining repair of targeted double-stranded DNA breaks.

In particular, AdV delivery of eCas9.4NLS into muscle progenitor cells resulted in significantly higher editing frequencies at defective DMD alleles causing Duchenne muscular dystrophy (DMD) than those achieved by AdVs encoding the parental, eCas9.2NLS, protein. In conclusion, this work provides a strong rationale for integrating viral vector and optimized gene-editing technologies to bring about enhanced RGN delivery and performance.

The NLS is a critical element as pointed out by Zhang in the Patent. In addition the work Kosugi et al, Cokol et al, Cartier and Reszka, and Lange et al.

As Lange et al had noted:

The best understood system for the transport of macromolecules between the cytoplasm and the nucleus is the classical nuclear import pathway. In this pathway, a protein containing a classical basic nuclear localization signal (NLS) is imported by a heterodimeric import receptor consisting of the β-karyopherin importin β, which mediates interactions with the nuclear pore complex, and the adaptor protein importin α, which directly binds the classical NLS. Here we review recent studies that have advanced our understanding of this pathway and also take a bioinformatics approach to analyze the likely prevalence of this system in vivo. Finally, we describe how a predicted NLS within a protein of interest can be confirmed experimentally to be functionally important.

8.3 Delivery Systems

As Yin et al note:

Efficient genome editing with Cas9–sgRNA in vivo has required the use of viral delivery systems, which have limitations for clinical applications.

Translational efforts to develop other RNA therapeutics have shown that judicious chemical modification of RNAs can improve therapeutic efficacy by reducing susceptibility to nuclease degradation.

Guided by the structure of the Cas9– sgRNA complex, we identify regions of sgRNA that can be modified while maintaining or enhancing genome-editing activity, and we develop an optimal set of chemical modifications for in vivo applications.

Using lipid nanoparticle formulations of these enhanced sgRNAs (e-sgRNA) and mRNA encoding Cas9, we show that a single intravenous injection into mice induces >80% editing of Pcsk9 in the liver. *Serum Pcsk9 is reduced to undetectable levels, and cholesterol levels are significantly lowered about 35% to 40% in animals. This strategy may enable non-viral, Cas9-based genome editing in the liver in clinical settings….*

Using the structure of Cas9–sgRNA complex as a guide, we have identified a number of design criteria that were transferable between different sgRNAs, highlighting the significance of the crystal structure to engineer chemically modified sgRNA. By avoiding modification of 2'OH and phosphate groups in the sgRNA that interact with Cas9 protein, chemical modifications can be made extensively, with ~70% of nucleotides chemically modified. This generalized approach may also be applied to direct comprehensive chemical modifications of sgRNA for other Cas9 proteins, for example, saCas9, or crRNA in CRISPR–Cfp1.

Our data also suggest that those 2'OH groups of sgRNA that interact with Cas9 by hydrogen bonding play a key role in the formation of a functional Cas9–sgRNA complex. This is distinct from the RNAi machinery, which does not require the 2'OH of siRNA to activate the RNase activity of RISC complexes.

Further studies will be needed to further optimize the modification pattern and to uncover potential sequence-specific effects. Non-viral genome editing is particularly attractive in a therapeutic setting, given the potential advantages of non-viral delivery systems, including ease of scale-up, speed of customization, lack of pre-existing immunity, and the possibility for limiting exposure to nuclease, among other items14,20. Here we demonstrate that appropriate chemically modified sgRNA enables very efficient in vivo (up to >80%), non-viral vector-mediated genome editing. A number of factors complicate accurate quantification: (1) larger-deletion-bearing alleles will possibly amplify more efficiently, although two differently sized PCR amplicons showed similar deletion of a 55-bp genomic sequence (Supplementary Fig. 3). (2)

The LNP used here is largely hepatocytespecific30, but only ~70–80% percent of the cells in a mouse liver are hepatocytes38. Since a large portion of hepatocytes are polyploid39, we estimate that hepatocytes account for >80% of total genome copies in the liver38,39 and show that the majority of gene editing occurred in hepatocytes. The LNP-mediated co-delivery of Cas9 mRNA and e-sgRNA (LNP–CRISPR) successfully depleted a disease-related protein through creating

high levels of indels at the corresponding genomic locus, suggesting its potential for disease treatment. We note that LNP-mediated siRNA therapy has been evaluated in phase 3 trials and the results are positive20. Non-viral genome editing with Cas9 has a number of potential advantages compared with siRNA therapies.

First among these, is that a single dose of LNP– CRISPR may provide for long-term therapeutic effects or potentially curative therapy, decreasing or likely eliminating the need for repeated injections. *Non-viral delivery of Cas9 as mRNA limits the exposure of the genome to Cas9, decreasing the potential for side effects relative to viral systems where the Cas9 gene is present for months or longer. A number of diseases demand correction or knock-in of sequences12. The applications of e-sgRNA to HDR and/or homology-independent targeted integration (HITI) are worth further investigations40. Beyond LNP delivery, we believe that these same types of modifications may facilitate delivery of e-sgRNA as a ligand-conjugate form without the need for encapsulation*

Wilbie et al note:

The discovery of CRISPR/Cas has revolutionized the field of genome editing. CRIPSR/Cas components are part of the bacterial immune system and are able to induce double-strand DNA breaks in the genome, which are resolved by endogenous DNA repair mechanisms. The most relevant of these are the error-prone nonhomologous end joining and homology directed repair pathways.

The former can lead to gene knockout by introduction of insertions and deletions at the cut site, while the latter can be used for gene correction based on a provided repair template. In this Account, we focus on the delivery aspects of CRISPR/Cas for therapeutic applications in vivo. Safe and effective delivery of the CRISPR/Cas components into the nucleus of affected cells is essential for therapeutic gene editing.

These components can be delivered in several formats, such as pDNA, viral vectors, or ribonuclear complexes.

In the ideal case, the delivery system should address the current limitations of CRISPR gene editing, which are (1) lack of targeting specific tissues or cells, (2) the inability to enter cells, (3) activation of the immune system, and (4) off-target events.

To circumvent most of these problems, initial therapeutic applications of CRISPR/Cas were performed on cells ex vivo via classical methods (e.g., microinjection or electroporation) and novel methods (e.g., TRIAMF and iTOP). Ideal candidates for such methods are, for example, hematopoietic cells, but not all tissue types are suited for ex vivo manipulation. For direct in vivo application, however, delivery systems are needed that can target the CRISPR/Cas components to specific tissues or cells in the human body, without causing immune activation or causing high frequencies of off-target effects. Viral systems have been used as a first resort to transduce cells in vivo. These systems suffer from problems related to packaging constraints, immunogenicity, and longevity of Cas expression, which favors off-target events. Viral vectors are as such not the best choice for direct in vivo delivery of CRISPR/Cas. Synthetic vectors can deliver nucleic acids

as well, without the innate disadvantages of viral vectors. They can be classed into lipid, polymeric, and inorganic particles, all of which have been reported in the literature. The advantage of synthetic systems is that they can deliver the CRISPR/Cas system also as a preformed ribonucleoprotein complex. The transient nature of this approach favors low frequencies of off-target events and minimizes the window of immune activation.

Moreover, from a pharmaceutical perspective, synthetic delivery systems are much easier to scale up for clinical use compared to viral vectors and can be chemically functionalized with ligands to obtain target cell specificity.

The first preclinical results with lipid nanoparticles delivering CRISPR/Cas either as mRNA or ribonucleoproteins are very promising. The goal is translating these CRISPR/Cas therapeutics to a clinical setting as well. Taken together, these current trends seem to favor the use of sgRNA/Cas ribonucleoprotein complexes delivered in vivo by synthetic particles.

Lino et al note:

8.3.1 Adeno-associated virus (AAV)

AAV, of the Dependovirus genus and Parvoviridae family, is a single stranded DNA virus that has been extensively utilized for gene therapy AAV is an excellent vehicle for gene therapy for many reasons. AAV is not known to cause or relate with any diseases in humans. There is also a wide range of known serotypes which allow for infection of a multitude of cells with different specificities. The virus itself is able to efficiently infect cells while provoking little to no innate or adaptive immune response or associated toxicity, at least upon first treatment with a serotype

8.3.2 Lentivirus (LV) and adenovirus (AdV)

While LVs and AdVs are clearly distinct, the way they are utilized for delivery of CRISPR/Cas9 components is quite similar. In the case of LV delivery, the backbone virus is a provirus of HIV (Naldini et al., 1996); for AdV delivery, the backbone virus is one of the many different serotypes of known AdVs. As in the case of AAV, these are plentiful, and finding a useful AdV to a desired target is relatively facile. The serotype most commonly used is AdV type 5. LV is particularly useful because it can be pseudotyped with other viral proteins, such as the Gprotein of vesicular stomatitis virus. In doing so, the cellular tropism of the LV can be altered to be as broad or narrow as desired. And, to improve safety, second- and third-generation LV systems split essential genes across three plasmids, significantly reducing the likelihood of accidental reconstitution of viable viral particles within cells. Both LV and AdV can infect dividing and non-dividing cells; however, unlike LV, AdV does not integrate into the genome.

8.3.3 Lipid nanoparticles/liposomes

Lipid nanoparticles have long been used as delivery vehicles for a wide range of different molecules to cells and have demonstrated popularity for nucleic acid delivery. Nucleic acids are typically unstable outside of cells, and owing to their highly anionic nature, they do not easily pass through the cell membrane. However, by encapsulating nucleic acids within typically very

cationic liposomes, they can be delivered to cells with relative ease. Lipid nanoparticles do not contain any viral components, which helps minimize safety and immunogenicity concerns. They can also, like viral particles, be utilized in vitro, ex vivo, and in vivo, allowing for extensive testing on a variety of scales of cell populations

8.3.4 Cell-penetrating peptides (CPPs)

CPPs are generally short stretches of amino acids that are polycationic, amphipathic, or non-polar in nature. Each class of CPPs can facilitate uptake of different types of proteins into different cell types, and often different combinations of CPPs and the desired molecule for uptake will result in different uptake levels. CPPs can be used for in vitro and ex vivo work quite readily, and extensive optimization for each cargo and cell type is usually required. Because of the level of detail required for this optimization, CPPs are not generally currently utilized to deliver components in vivo. In the specific case of CRISPR/Cas9, the CPPs are usually covalently attached to the Cas9 protein directly, which is then complexed with the sgRNA and delivered to cells.

8.3.5 DNA nanoclew

A DNA 'nanoclew' is a unique technology for CRISPR/Cas9 component delivery. Developed by Sun et al. (2014), a DNA nanoclew is a sphere-like structure of DNA that has been compared with a ball of yarn. The nanoclew is synthesized by rolling circle amplification with palindromic sequences that aide in the self-assembly of the structure. The sphere can then be loaded with a payload – Sun et al. originally used doxorubicin – and the payload can be specifically triggered for release by certain biological conditions. As this is a relatively new delivery technology, it has currently only been utilized in an in vitro setting.

8.3.6 Gold nanoparticles (AuNPs)

AuNPs have many uses in applied biomedical science, from imaging agents to inert carriers of other components. As such, these particles are readily used in in vitro, ex vivo, and in vivo settings. Mout et al. (2017) demonstrated that, by engineering Cas9:sgRNA RNP and AuNPs to associate with one another (Figure 8(B)), a complex is created that can be efficiently delivered to cells and cause a desired mutation at a rate of roughly 30%. Lee et al. (2017) also reported use of AuNPs to deliver Cas9:sgRNA RNP to mice suffering from DMD. In this work, 15 nm diameter AuNPs were conjugated to thiolated short DNA oligos (Figure 8(C)), which were then conjugated to a single-stranded donor DNA. This donor DNA then complexed with the Cas9 RNP. The resulting particle was coated in a silicate and an endosomal disruptive polymer, PAsp(DET). A single injection of the AuNP-Cas9 conjugate corrected 5.4% of the mutated DMD-causing dystrophin gene and showed recovered dystrophin gene expression. Treated mice further showed partial restoration of muscle function and reduced levels of fibrosis.

8.3.7 Lipid-coated mesoporous silica particles

Developed by Brinker and colleagues at Sandia National Laboratories and the University of New Mexico, this biological delivery system is composed of a mesoporous silica nanoparticle core and a lipid membrane shell (Liu et al., 2009). While not yet utilized for CRISPR/Cas9, the particles have intriguing properties that may make them good delivery vehicles for the technology. The silica core has a large internal surface area, leading to high cargo loading capacities. In addition, pore size, pore chemistry, and overall particle size can be individually tailored, allowing for the loading of various types of cargo (Du et al., 2014; Durfee et al., 2016). The lipid coating of the particle can also be tailored to maximize cargo loading, increase circulation times, and provide precise targeting and cargo release.

8.3.8 Inorganic nanoparticles

Inorganic nanoparticles are natural potential CRISPR component carriers because they have already been used for similar purposes. Examples of these include AuNPs, carbon nanotubes (CNTs), bare mesoporous silica nanoparticles (MSNPs), and dense silica nanoparticles (SiNPs). The use of AuNPs for CRISPR/Cas9 delivery was described above. While CNTs, MSNPs (Luo et al., 2014), and SiNPs (Luo and Saltzman, 2000) have been used for many gene delivery applications, the use of these carries for Cas9 delivery has yet to be reported.

8.3.9 Physical Transfection

Fajrial et al have noted:

Recently, novel physical methods for transfection have surged due to the development of microtechnology and nanotechnology. Nanostructure-mediated electroporation, for example, allows miniaturization of physical transfection to improve transfection efficiency and precision. It uniformly treats cells with minimal damage to cell viability in comparison to stochastic uneven cell permeabilization using bulk electroporation. Physical transfection does not rely on the use of vectors. Consequently, unlike viral vectors, there is almost no limit to cargo size, and unlike chemical vectors, the rate-limiting step does not depend on cell endocytosis. … physical transfection can harness energy from electrical, thermal, and mechanical forces. Applied forces compromise the cell membrane, allowing the cargo to diffuse into the cell and, in some cases, assist in active delivery of the cargo itself. Electroporation, for example, shocks the cell with an electric field that induces membrane perforation and drift forces to charged cargo such as plasmid DNA.

Despite the advantages of physical transfection, there are also limitations. In vivo physical transfection is still too invasive for human application, although special circumstances warrant further exploration. DNA vaccines, whose delivery technique is identical to gene editing, have been physically transfected in vivo into mice using gene gun injection and electroporation. DNA vaccine only requires intradermal or intramuscular delivery unlike many gene therapies for genetic diseases that are generally invasive.

For gene editing, in vivo electroporation has demonstrated the delivery of genetic materials into retina and epidermis tissue in mice. Similarly, a silicon nanoneedle can deliver plasmid DNA encoding the vascular endothelial growth factor into the muscles of mice, promoting tissue

neovascularization. However, due to its invasive nature, in vivo transfection usually involves delivery vectors.

8.4 Adverse Effects

Finally we always address adverse effects. The more accurate the targeting the better the chance that adverse effects can be mitigated. As Mehta and Merkel note:

Clustered regularly interspaced short palindromic repeats (CRISPR) form the adaptive immune system in archaea and bacteria and have been modified for genome engineering in eukaryotic cells. CRISPR systems contain 2 components, a single-guide RNA, which is a short RNA composed of a 20 nucleotide sequence that targets specific sites in the genomic DNA and a scaffold necessary for its binding to the CRISPRassociated endonuclease (Cas9).

Because of its high efficiency and accuracy, the CRISPR-Cas9 genome editing based therapies are poised to treat a multitude of human diseases with a promise to target previously "undruggable" proteins. As the first in-body clinical trial with CRISPR-Cas9 is embarked on, the risks associated with administering the genome editing machinery to patients become increasingly relevant. Recent studies have demonstrated an innate and adaptive cellular immune response to Cas9 in mouse models and the presence of anti-Cas9 antibodies and T-cells in human plasma.

Pre-existing immunity against therapeutic Cas9 delivery could decrease its efficacy in vivo and may pose significant safety issues. This review focuses on the immunogenicity of the Cas9 protein and summarizes potential approaches to circumvent the problem of immune recognition.

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10 INDEX