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# Discovery of the PTEN Tumor Suppressor and Its Connection to the PI3K and AKT Oncogenes

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PTEN (phosphatase and tensin homolog on chromosome 10) was discovered over 20 years ago in 1997 and linked to the phosphatidylinositol 3-kinase (PI3K) and AKT oncogenes the following year. The discovery of PTEN emerged from the linked concepts of oncogenes and tumor suppressor genes that cause and prevent cancer and the fields of tumor viruses and human cancer genetics from which these two concepts arose. While much has been learned since, the initial discovery and characterization, including the discovery that PTEN is a regulator of PI3K and AKT, provide the foundation on which we continue to build our knowledge. To provide the context in which these cancer genes were discovered, background information that led to their discovery will also be discussed, which will hopefully be a useful guide for readers seeking to build on the work of others.

## THE ONCOGENE CONCEPT AND EVIDENCE TO SUPPORT IT

The oncogene concept emerged from the tumor virus field, which developed over a 60-year period from the discovery of the first cancer-causing virus in chickens at the beginning of the twentieth century (Rous 1911). By the 1950s, it had been established that there were many different independent types of mammalian viruses that could cause cancer (Gross 1974). Their names were often eponymous and a consequence of their species tropism and the type of cancer that they caused. By the 1960s, it was clear that polynucleotides from the different viruses carried the genetic information needed to infect and transform cells, and tumor viruses were broadly divided into the DNA and RNA tumor viruses based on the type of polynucleotide packaged in the virus particle (Javier and Butel 2008). The idea that genes could cause cancer was an extremely old one (Rous 1911), but the evidence was difficult to acquire because it required the isolation of the viral gene that was capable of causing a tumor, which was dependent upon technological innovation. The technology to measure the ability of viral particles and their clonal progeny to cause cellular transformation in tissue culture was developed in the 1960s, which allowed for viral cloning and characterization of their ability to cause cancer (Dulbecco 1960). With isolated cloned viruses in hand, the new technologies of DNA restriction enzymes and bacterial cloning, which were developed in the 1970s, allowed for the isolation of the viral genes that caused cancer. In 1975, the

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first oncogene was isolated by three groups who independently discovered that the "*A* gene" of SV40 virus, which had been determined to encode the large T-antigen a few years earlier, was the cancer-causing gene (Brugge and Butel 1975; Martin and Chou 1975; Tegtmeyer 1975). The proof that the gene encoding large T-antigen was the SV40 oncogene was obtained through the mapping of the SV40 A gene to a temperature-sensitive mutation that was able to conditionally cause cellular transformation at the permissive temperature and revert to normal morphology at the higher nonpermissive temperature. Further mapping determined that the SV40 gene encoding small T-antigen was also an oncogene. Analysis of the polyoma virus oncogenes showed that like SV40 it also had small and large T-antigens; however, in addition, the virus had an additional potential oncogene encoding middle T-antigen (Lania et al. 1979; Treisman et al. 1981).

A similarly behaving temperature-sensitive mutation of Rous sarcoma virus was identified in 1970 (Martin 1970), but because of the difficulty of mapping genes to RNA, it was not until 1976 that the mutation could be mapped to a specific gene (Bernstein et al. 1976). In the same month, another group used an entirely different method for mapping the oncogene, termed *src*, for the virus by showing that the transforming form of the virus contained genetic information homologous to chicken and other avian species, which provided the first evidence that cellular genetic information could be oncogenic in viruses (Stehelin et al. 1976).

In 1982, the first oncogene derived from mammalian cells and not a virus was cloned from human cancer cells by isolation of a DNA fragment capable of transforming immortal mouse fibroblasts (Cox and Der 2010). It was recognized that the transforming human gene was homologous to the transforming gene from the rat sarcoma virus genome and contained similar point mutations. Two forms of *ras* oncovirus (Harvey and Kirsten) were found to have distinct human homologs. Excitingly, the *KRAS* oncogene was somatically mutated at high frequency in human colon cancer. In a similar time frame, human homologs of other RNA tumor virus oncogenes such as *myc*, *abl*, and *erbb1* and *erbb2*, were found also to be mutated into active forms by translocation and amplification that occurred in the tumor cell. The above advances were aided by new technologies for genetics and molecular biology including gel electrophoresis, Southern and northern blotting, reverse transcriptase, restriction enzymes, recombinant cloning in bacterial vectors, and DNA sequencing.

One of the pressing questions of the time was to understand how these oncogenes were able to cause cancer. Pertinent to this story, one of the first insights came from work with polyoma middle T-antigen. Antibodies to middle T-antigen were able to coprecipitate *src* protein and its associated tyrosine kinase activity from cells (Smith et al. 1979; Courtneidge and Smith 1983). This was an exciting finding since it was the first to link the DNA tumor virus oncogene to an oncogenic protein from an RNA tumor virus. Antibodies to middle T-antigen were also shown to coprecipitate with another enzyme activity able to phosphorylate the lipid phosphatidylinositol in vitro (Whitman et al. 1985). Middle T-antigen's transformation activity was dependent on its ability to interact with the lipid kinase activity (Kaplan et al. 1986) and soon thereafter a human cellular phosphatidylinositol kinase activity was isolated that was distinct from *src* and middle T-antigen, which specifically phosphorylated the third alcohol of phosphatidylinositol 4,5-bisphosphate on the lipid's inositide head group, thus leading to the name phosphatidylinositol 3-kinase (PI3K) (Whitman et al. 1987, 1988).

## THE TUMOR SUPPRESSOR CONCEPT AND EVIDENCE TO SUPPORT IT

Like the idea of the oncogene, the concept of the tumor suppressor is over a century old (Hansford and Huntsman 2014). The first functional evidence for tumor suppression came from cell fusion experiments, which showed that normal cells fused with cancer cells could suppress cancer formation (Harris et al. 1969). However, Knudson developed the concept further in his effort to understand the basis for the difference

between early- and late-onset pediatric retinoblastoma by positing that the early-onset disease was associated with inheritance of a mutant autosomal allele followed by loss of the wild-type allele at the time of tumor initiation and that the late-onset form required that both copies were altered after birth (Knudson 1971). However, the precise localization of a tumor suppressor gene had to await the mapping to the gene responsible for retinoblastoma. Karyotypes from patients with retinoblastoma revealed that some patients had small deletions within chromosome 13q (Orye et al. 1974). Use of DNA fragments mapping to chromosome 13 revealed that loss of heterozygosity (LOH) could be detected in retinoblastoma samples compared to normal DNA from the same patient (Cavenee et al. 1983). In 1986, a key paper that was the culmination of years of effort to isolate and locate the gene responsible for retinoblastoma (*RB1*) was published (Friend et al. 1986). Importantly, unlike the previously mentioned oncogenes that are defined by their ability to cause cancer, the RB1 gene was the first example of a tumor suppressor gene, which is defined by its ability to guard against and suppress cancer development. Restoration of RB1 into retinoblastoma cell lines was later shown to suppress cancer growth (Huang et al. 1988). The location of the responsible gene was made possible by mapping from retinoblastoma cases a region of minimal LOH to identify the approximate gene locus followed by mapping of smaller homozygous deletions where both copies of DNA were deleted. The DNA fragments that were only present in wild-type cells were used to identify and clone the RB1 transcript by hybridizing them to a human cDNA library cloned into bacteriophage. In 1987, the encoded RB1 protein was found to encode a protein of 110 kDa (Lee et al. 1987a, 1987b).

Like SV40 and polyomavirus, adenovirus, another DNA tumor virus, was found to encode oncogenes, one of which was E1A. Ed Harlow at Cold Spring Harbor and his Stony Brook graduate students determined that an antibody to E1A could coprecipitate with a cellular 110 kDa protein (Harlow et al. 1986). Soon thereafter, they determined that this protein was the RB1 protein, which was the first time a viral oncoprotein was found to interact with a tumor suppressor protein (Whyte et al. 1988). Soon thereafter, SV40 large T-antigen was found to interact with RB1 as well (DeCaprio et al. 1988).

Another very enlightening discovery, which occurred the following year, was the demonstration that p53, another cellular protein-binding partner of SV40 large T-antigen, an interaction that was first identified in 1979 by multiple groups, was encoded by a tumor suppressor gene (Levine and Oren 2009). Mapping of LOH in colon cancer identified a minimal region of loss where the gene for p53 was located on chromosome 17p. Sequencing of two colon cancers having a loss of one allele determined that the remaining allele had a p53 mutation (Baker et al. 1989). Functional studies showed that wild-type p53 suppressed tumor cell growth (Baker et al. 1990). Soon thereafter, it was determined that the p53 gene was mutated in affected individuals in families with the Li–Fraumeni cancer predisposition syndrome (Srivastava et al. 1990). Thus, it was now apparent that the DNA tumor virus transformed normal cells at least in part by disrupting two tumor suppressor proteins RB1 and p53.

## IDENTIFICATION OF THE PI3K/PTEN PATHWAY

### Tumor Viruses and the Discovery of the PI3K and AKT Oncogenes and Oncoproteins

The foundational discovery identifying and showing that the PI3K pathway was activated in cancer came from the study of immunoprecipitates of the polyoma middle T-antigen oncoprotein that were found to coprecipitate with a kinase activity capable of phosphorylating phosphatidylinositols by the mid-1980s. Further characterization of mutations of middle T-antigen determined that the interaction with the inositol kinase activity was required for it to behave as an oncogene. Analysis of the new enzyme determined that it specifically phosphorylated the third position of the inositol ring of the plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate and that it had

two components, one of 110 kDa that was the catalytic subunit and another of 85 kDa that was the regulatory subunit, that were encoded by separate genes, now known as PIK3CA and PIK3R1 (Whitman et al. 1988; Carpenter et al. 1990). Based on these features, the enzyme was named PI3K. Moreover, the kinase was determined to be activated rapidly within seconds in response to a wide range of extracellular signals including insulin and platelet-derived growth factor (PDGF), which led to the accumulation of its product phosphatidylinositol-3,4,5-trisphosphate ($PIP_3$), which was short-lived, and presumed to act as a second messenger to signal to other proteins (Whitman et al. 1987). PI3K, which is known as PI3Kα, was the first member of the large family of enzymes in this class including the closely related PI3Kβ, PI3Kγ, PI3Kδ, and more distantly related mTOR and ATM, among others. Independent evidence that PI3Kα acts an oncogene came from multiple sources including the isolation of PIK3CA, which encodes the p110α catalytic subunit, as a chicken retrovirus oncogene in 1997 (Chang et al. 1997), and the later discovery of mutations of human PIK3CA, which often occurred in common cancers at rates that are very high, particularly in breast cancer (Campbell et al. 2004; Samuels et al. 2004; Saal et al. 2005). Importantly, these mutations not only activated the enzyme, they also could transform cells and cause cancer in different model systems with the most common hotspot mutations occurring in PIK3CA at codons 542, 545, and 1047 (Bader et al. 2006). In addition to being activated through extracellular signals, the p110 kDa catalytic subunit has an RAS-binding domain through which RAS oncoproteins can bind and activate PI3K directly. PIK3CA mutations are most commonly found in estrogen receptor (ER)-positive breast cancer (Saal et al. 2005), and inhibition of PI3Ka in these tumors with a PI3Kα selective inhibitor in combination with inhibition of the ER has recently been shown to have benefit for patients with metastatic cancer with this genotype (Juric et al. 2019).

The AKT8 mouse RNA tumor virus was first isolated in 1977 by Stephen Staal from a strain of AKR mice prone to spontaneous lymphoma. Ten years later, in 1987, he sequenced the viral genome and identified that it contained a putative oncogene that was homologous to two human genes, of which one, AKT1, was amplified in a subset of some human cancers. Soon thereafter, AKT2 was also found to be amplified in other cancer types (Bellacosa et al. 1995; Cheng et al. 1996). AKT was discovered to be a serine–threonine protein kinase in 1991 (Bellacosa et al. 1991) and shown to be regulated by PI3K (Franke et al. 1995). The molecular link between PI3K and AKT was determined in 1997 when it was shown that the PH domain of AKT could bind specifically to the product of PI3K, $PIP_3$, which was required for plasma membrane binding and activation of the kinase activity of AKT (Franke et al. 1997). Interestingly, a hotspot oncogenic mutation of AKT1 (E17K) alters the PH domain pocket, thus bringing the mutant protein to the plasma membrane (Carpten et al. 2007).

The AKT kinase has a wide range of target proteins within the cell that have varied physiological and oncogenic effects, which is beyond the scope of this review. However, one of the main AKT targets involved in cancer is the mTOR protein complex TORC1, which is inhibited by the TSC1 and TSC2 tumor suppressors. Activated AKT phosphorylates TSC2, which leads to the degradation of the TSC1/TSC2 complex, as well as the TORC1 subunit PRAS40, and allows for activation of TORC1 kinase, which has a variety of progrowth effects on the cell that stimulate lipid, nucleotide, and protein synthesis (Inoki et al. 2002; Manning et al. 2002; Tee et al. 2002).

## Tumor Suppressor Gene on Chromosome 10

As discussed above, the concept of tumor suppressor genes emerged from the field of human cancer genetics that mapped specific germline and somatic chromosome alterations in early-onset cancer predisposition syndromes such as retinoblastoma and the field of tumor cell biology that showed that fusion of normal cells with cancer cells or expression of a tumor suppressor gene from an expression vector could suppress cancer cell growth. The discovery of the RB1

tumor suppressor in 1986 using these technologies elicited a large-scale search for other tumor suppressor genes.

Studies of the karyotypes of glioblastoma multiforme tumor cell lines by Darryl and Sandra Bigner at Duke University determined that these cells lost one copy of chromosome 10 at a frequency that was much higher than seen for other chromosomes (Bigner et al. 1984). Analysis of allelic losses by the Cavenee and Bigner laboratories in 1988 comparing glioblastoma biopsies and normal tissue DNA determined that LOH for chromosome 10 occurred at high rates in glioblastoma but was not seen in lower grade astrocytomas (Bigner et al. 1988; James et al. 1988). Mapping studies to locate a minimal region of chromosomal loss implicated the long arm of chromosome 10 (Rasheed et al. 1992). Inspired by these genetic mapping studies in glioblastoma samples, normal chromosome 10 from fibroblasts was fused to glioblastoma cell lines, which suppressed tumor cell growth in soft agar and in mice; control experiments that fused chromosome 2 did not suppress tumor growth (Pershouse et al. 1993).

The relevance of chromosome 10 to other types of cancer was soon realized. An analysis of prostate cancer allelic loss patterns determined that loss of chromosome 10q was a frequent occurrence (Gray et al. 1995). This was soon followed by a linkage analysis study that determined that the hereditary cancer predisposition syndrome known as Cowden disease (or syndrome) was linked to chromosome 10q22–23 (Nelen et al. 1996), a segment contained within a previously implicated region found in tumor LOH studies. Cowden syndrome is associated with increased risk for the development of benign neoplasms known as hamartomas, but also cancer, including cancers of the breast and thyroid (see Ngeow and Eng 2019).

Based upon the high frequency of chromosome losses in glioblastoma and prostate cancer and the linkage to the cancer predisposition syndrome Cowden syndrome, there was a high index of suspicion that chromosome 10 harbored one or more tumor suppressor genes that might be broadly relevant to multiple types of human cancer. Efforts to isolate the gene using positional cloning methodologies were undertaken in multiple laboratories. One approach to isolate the gene was to use cell fusions that contained chromosome 10 fragments to identify the minimal region able to suppress tumor cell growth (Steck et al. 1995). Another approach was to use microsatellites to map a minimal region of LOH. Both of these approaches were limited in resolution and led to multiple different candidate regions containing a possible tumor suppressor gene.

Alternatively, mapping of tumor homozygous deletions, which are much smaller than heterozygous deletions, as a means to isolate a relatively small region containing only one gene was a powerful method for identifying tumor suppressor genes and had been previously used for the identification of the RB1, CDKN2A, and SMAD4 tumor suppressor genes (Friend et al. 1986; Kamb et al. 1994; Hahn et al. 1996). Therefore, it seemed reasonable to scan the whole genome for homozygous deletions in cancer samples utilizing a genomic subtraction method known as representational difference analysis that identified probes present in a normal genome but not in the matched tumor (Lisitsyn et al. 1993). Probes identified from the subtraction were mapped using radiation hybrids to different parts of the genome and tested for recurrent homozygous deletion in other cancer cases (Li et al. 1997). One of the genomic probes representing a possible homozygous deletion in a breast cancer mapped to the 10q23 region of LOH and linkage identified in glioblastoma, prostate, and Cowden syndrome. Mapping of allelic losses in breast cancer also determined that chromosome 10q23 was a region of LOH in nearly 40% of cases; however, this initial probe was not able to identify homozygous deletions in any of the samples tested (Li et al. 1997).

The key genomic means for cloning PTEN was identified by cataloging as many probes as possible in the 10q23 region and examining them for recurrent homozygous deletion in a panel of 65 breast cancer cell lines and xenografts (Li et al. 1997). One probe from this region AFMA086WG9 was homozygously deleted in two xenografts from these 65 cases. When it was tested in prostate and glioblastoma samples,

it was also completely missing in multiple glioblastoma and prostate cancer cell lines and glioblastoma xenografts. The probe was then used to isolate multiple bacterial artificial chromosome plasmids. These large plasmids were used to generate new probes, which were then used to generate a higher resolution probe map of the region. One of the BACs was then also used to make an exon trapping library in a minigene plasmid containing a cloning site between splice donor and splice acceptor exons that was transfected into cells from which RNA was extracted and cDNA prepared for polymerase chain reaction (PCR) to detect possibly trapped exons. Two distinct exons were isolated and the DNA was sequenced. The exon sequence was entered into a BLAST search and was found to be 100% identical to a series of random cDNA clones from different human tissues. The full-length cDNA was isolated and assembled by sequencing and analyzing a large series of independent cDNA clones. Based upon the predicted 403 amino acid open reading frame of the transcript, there was strong homology with the catalytic pocket of many dual-specificity tyrosine and serine/threonine phosphatases with the greatest homology with CDC14. Moreover, the phosphatase domain was within a larger region of the protein that had strong homology with the protein tensin. Examination of other species' DNA sequence available at the time showed that PTEN had a homolog in *Saccharomyces cerevisia*. Because of its homology with phosphatases and tensin and its location on chromosome 10, the gene was named PTEN.

To obtain genetic evidence that PTEN was the target of the deletions and the candidate tumor suppressor, the exon trap and the BAC probes were used to determine whether any of the homozygous deletions were intragenic. Several homozygous deletions proved to be intragenic by this approach with one in the glioblastoma cell line DBTRG-05 occurring within one of the exon probes. Sequence analysis of the cDNA and exons determined that many of the tumor cell lines were, in fact, harboring missense, nonsense, and frameshift mutations. Examination of glioblastoma samples from patients also showed many similar mutations in the tumor, which were not present in normal tissue matched to the same patient. Analysis of germline DNA from Cowden disease affected family members that they had *PTEN* mutations affecting the predicted open reading frame in their germline (Liaw et al. 1997). A parallel effort using similar approaches by another group independently identified the PTEN tumor suppressor gene with a demonstration of mutation in a broad range of advanced cancers, which led to their naming the gene, *MMAC1*, for mutated in multiple advanced cancers 1 (Steck et al. 1997).

While the initial papers documented somatic mutation of *PTEN* in a wide range of tumor types, follow-up papers by these groups and others rapidly cataloged the range of different tumors and the point in tumor progression when PTEN became mutated. These findings documented that PTEN is mutated in glioblastoma but not lower grade astrocytomas (Wang et al. 1997; Duerr et al. 1998), prostate carcinoma (Wang et al. 1998), breast cancer (Bose et al. 1998), and endometrial carcinoma (Tashiro et al. 1997). Mutation of PTEN was also documented in other human malignancies including lymphoma and cancer of the bladder, head and neck, ovary, thyroid, and gastrointestinal system but at lower frequency (see Ngeow and Eng 2019).

Early biochemical and biological efforts showed that PTEN was a protein phosphatase with selectivity toward negatively charged polypeptide substrates that required this catalytic function to inhibit cell proliferation and activate cell death when it was introduced into cancer cell lines (Furnari et al. 1997; Myers et al. 1997; Li et al. 1998). It was also quickly determined that the PTEN gene protein product was a lipid phosphatase that was able to remove the third inositol phosphate from the substrate $PIP_3$, the product of PI3K (Maehama and Dixon 1998; Myers et al. 1998; Stambolic et al. 1998). Absence of functional PTEN in cells led to elevated levels of $PIP_3$, which activated AKT kinase, and restoration of PTEN using expression vectors lowered $PIP_3$ and AKT signals. Moreover, cell death induced by PTEN in mutant breast or prostate cancer cell lines could be rescued by expression of myrisoylated-AKT, and the ex-

pression of PTEN in a glioblastoma cell line with mutant PTEN inhibited cell proliferation, which was associated with altered gene expression similar to the pattern seen with an inhibitor of PI3K (Li et al. 1998; Stolarov et al. 2001). Loss of PTEN also activated p70 S6 kinase (Podsypanina et al. 2001). Another function of PTEN that was rapidly appreciated was its ability to inhibit cell migration, which unlike its ability to regulate signaling (AKT, p70S6K) or the phenotypes of cell proliferation and cell death, did not require a functional PTEN phosphatase domain (Tamura et al. 1998). Last, in addition to its phosphatase domain, PTEN was found to have a C2 domain needed for membrane binding and a tail domain that was highly phosphorylated thereby interfering with interaction with the plasma membrane and increasing protein stability (Lee et al. 1999; Vazquez et al. 2000).

Genetic studies in mice were highly informative and showed that *pten*$^{+/-}$ heterozygous mice developed neoplastic lesions in many different organs including epithelial tumors of the prostate, mammary gland, colon, uterus, thyroid, liver, and adrenal gland, as well as hyperplasia of lymph nodes with abnormal expansion of B-cell and T-cell lineages and that these lesions showed evidence of increased phosphorylation and activation of AKT, mTOR, and p70S6K (Di Cristofano et al. 1998; Suzuki et al. 1998; Podsypanina et al. 1999; Stambolic et al. 2000). Because mTOR activated p70S6K kinase in the tumors from *pten*$^{+/-}$ mice, the mice were treated with a rapamycin analog, which lowered p70S6K activity as expected and also inhibited the proliferation of the neoplastic tumor cell lesions in these animals (Podsypanina et al. 2001).

The above studies, which were all published by 2001, form the foundation for the PTEN field. The findings established the identify to the chromosome 10 tumor suppressor, determined that it could regulate cell phenotypes like proliferation, migration, and death and acted as a phosphatase on a signal transduction pathway that could be down-regulated by either restoring PTEN expression or a small molecule inhibitor acting on a kinase including and downstream from PI3K. Research since 2001 has not only built on these findings but has also identified new and unanticipated functions for PTEN.

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