The Pezcoller Foundation

Journal

Summary

News from the
Pezcoller Foundation World

Year 23 - no 41
October 2013

October 2013

We have the great privilege to host in these pages the text of the last Korsmeyer Lecture by the prestigious winner of the 2013 Pezcoller Foundation-AACR International Award for Cancer Research prof. Peter K. Vogt, PhD, professor of Molecular Medicine at the Scripps Research Institute, La Jolla, CA.
Prof. Vogt gave the lecture entitled "Phosphatidylinositol-3 kinase (PI3K) and Cancer" at VIMM Venetian Institute of Molecular Medicine in Padua on May 8, two days before the Award Ceremony at the Buon Consiglio Castle in Trento and we express our gratitude for giving us the opportunity to publish it.
Please note that the annual Stanley J. Korsmeyer Lectureship has been started by the Pezcoller Foundation in 2006 in accordance with the AACR American Association for Cancer Research and the VIMM Venetian Institute of Molecular Medicine in Padua. The goal of this event is to honor the fundamental contribution of the late S. Korsmeyer who was the recipient of the Pezcoller Foundation-AACR International Award for Cancer Research in 2004. Although under heavy treatment for cancer, he presented his last European lecture at VIMM immediately before receiving the Pezcoller Award. Unluckily he passed away a few months later. Therefore we wish to remember Stanley Korsmeyer every year with a lecture given by the recipient of this Award.
The 2013 Pezcoller Symposium entitled "Metabolism and Tumorigenesis" took place in Trento last June with a large participation of researchers under the leadership of Drs. David Livingston (Dana Farber Cancer Institute, Boston); William Kaelin (Harvard Medical School, Boston); Massimo Loda (Harvard Medical School, Boston); Karen Vousden (Beatson Institute, Glasgow) and the support of Dr. Enrico Mihich (Dana Farber Cancer Institute, Boston).
During the session we gave the "Pezcoller Begnudelli Awards" for the best posters to Elena Favaro, Oxford University; Valentina Audrito, Turin University and Martina Chiu, Parma University.
We are also glad to present the next 26th Pezcoller Symposium which will be held in Trento on June 19-21, 2014 and will be entitled "CANCERS DRIVEN BY HORMONS".

On September 13 in Rovereto, in the wonderful Conference Hall of the Palazzo Istruzione (foto n. 027), we gave the Pezcoller Foundation-ECCO Recognition for Contribution to Oncology (€ 30.000,00) to Anita Margulies, an internationally known nurse, for her contribution to the field of oncology and for the subsequent impact on patient care, in particular through primarily evidence dissemination and implementation of best practice. Anita Margulies gave the Pezcoller Lecture at the 17 ECCO Congress on September 29 in Amsterdam.

Gios Bernardi M.D.
Editor and Pezcoller Foundation President Emeritus

Above: Anita Margulies, winner of the Pezcoller Foundation-ECCO Recognition for Contribution to Oncology and Davide Bassi president of the Pezcoller Foundation.

Picture on front page:
Peter K. Vogt

Phosphatidylinositol-3 kinase (PI3K) and Cancer[1]

Peter K. Vogt
The Scripps Research Institute, Department of Molecular and Experimental Medicine

A canonical signaling pathway. The enzyme phosphatidylinositol-3 kinase (PI3K) denotes a family of lipid kinases that phosphorylate the inositol ring of phosphatidylinositol at the 3' position (Fig. 1). There are three classes of PI3K that differ in substrate specificity and structure. Class I has been studied most extensively because of its role in oncogenesis. Class I PI3Ks are dimeric enzymes consisting of a catalytic subunit p110 that in the cell is bound to a regulatory subunit, the most common one being p85 (Fig. 2). The interaction with p85 stabilizes p110 and also inhibits its enzymatic activity. This inhibition is relieved by incoming upstream signals originating typically in active receptor

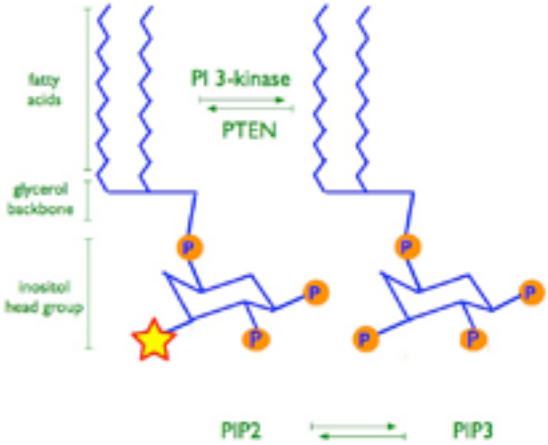

Fig. 1. The enzymatic action of class I phosphatidylinositol-3 kinase (PI3K). PI3K is a lipid kinase that phosphorylates the inositol ring of phosphatidylinositol at the 3' position. This reaction is catalyzed in the opposite direction by PTEN (Phosphatase and Tensin Homolog) which therefore acts as an antagonist of PI3K.

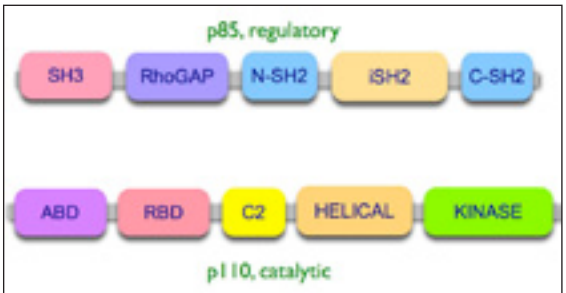

Fig. 2. PI3K is a dimeric enzyme, consisting of a regulatory subunit p85 and a catalytic subunit, p110. Both proteins are organized in structurally and functionally distinct domains. p85 contains several protein-protein interaction domains (nSH2, cSH2, SH3) that are important in the interaction with upstream signaling components and with p110. The iSH2 domain makes inhibitory contacts with p110. The function of the RhoGAP (Rho GTPase activating protein) domain in p85 is not understood. The catalytic subunit contains the ABD (adaptor binding domain) which is essential for binding to p85, and the adjacent RBD (RAS-binding domain) interacting with RAS. The C2 domain is the main target of the inhibitory contacts with p85. The C-terminal portion of p110 harbors the helical domain (function largely unknown) and the kinase domain.

[1]A condensed transcript of the Stanley Korsmeyer Memorial Lecture delivered on May 8, 2013 at the Venetian Institute of Molecular Medicine VIMM, Padova, Italy.

tyrosine kinases or G protein-coupled receptors. PI3K initiates complex cellular signals that affect a large number of activities including cellular replication. The canonical core pathway of PI3K signaling begins with the product of PI3K, PIP_3 (phosphatidylinositol-trisphosphate) (Fig. 3). PIP_3 recruits two serine/threonine kinases, AKT (cellular homolog of murine thymoma virus akt8 oncoprotein) and PDK1 (3-phosphoinositide-dependent protein kinase-1). PDK1 phosphorylates and thereby activates AKT. AKT has numerous downstream targets; for the purpose of this brief discussion we will ignore all except one, TSC2. TSC2 is a component of the tuberous sclerosis complex and functions as a GAP (GTPase activating protein) for RHEB (Ras homolog enriched in the brain). Phosphorylation of TSC2 by AKT inactivates the GAP activity of TSC2 resulting in elevated levels of GTP-bound RHEB. This activated RHEB interacts directly with the downstream serine/threonine kinase TOR, resulting in the activation of TORC1 (TOR complex 1), an aggregate of several proteins that besides TOR contains RAPTOR (regulatory-associated protein of mTOR) as its defining component. Active TORC1 stimulates protein synthesis by phosphorylating several downstream targets. This idealized and overly simplified signaling pathway contains several potentially oncogenic proteins. These include upstream components such as receptor tyrosine kinases, PI3K itself, AKT, RHEB and a downstream target of TORC1, eIF4E (eukaryotic initiation factor 4E). The pathway also contains important tumor suppressors, notably PTEN (phosphatase and tensin homolog), the enzymatic antagonist of PI3K and the TSC complex.

PI3K as oncoprotein. PI3K has long been connected to viral oncogenic activity. PI3K binds to the oncoproteins of DNA tumor viruses, and the gene encoding p110 functions as the sole oncogene in avian retrovirus ASV16 that induces rapidly growing hemangiosarcomas in chickens. In the genome of this virus, the p110 sequences have replaced most of the viral genetic information (Fig. 4). This virus is therefore defective in replication and depends on a

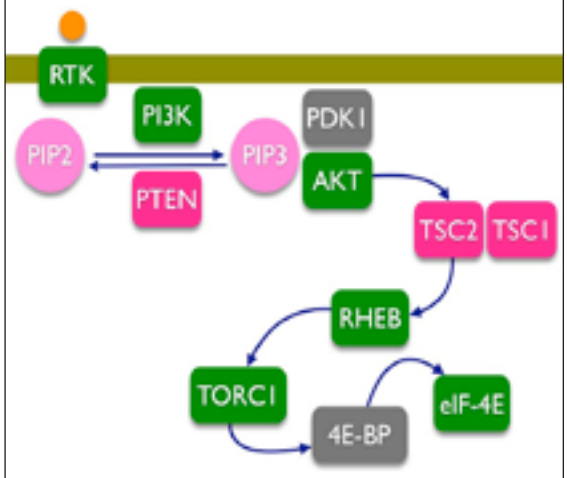

Fig. 3. A canonical signaling pathway of PI3K. PI3K is typically activated by upstream signaling activity that originates in a G-protein coupled receptor or (as in this figure) in a receptor tyrosine kinase. The product of PI3K, PIP_3, recruits AKT and PDK1, resulting in the activation of AKT. AKT phosphorylates and thereby inactivates TSC2 which results in an elevation of GTP-bound RHEB and activation of TORC1. TORC1 is an important positive regulator of protein synthesis.

Fig. 4. The genetic map of ASV16 (avian sarcoma virus 16). This replication-defective retrovirus encodes a single fusion protein that consists of N-terminal viral GAG sequences fused to the entire coding region of the alpha isoform of p110 of PI3K. The virus induces hemangiosarcomas in chickens, it propagates itself with the aid of a replication-competent helper virus that provides the missing viral functions for ASV16 in trans.

Fig. 5. Around 80% of the cancer-specific mutations of p110 occur in two hot spots in coding regions of the gene PIK3CA. One of the hot spots is located in the helical domain of p110 (cf. Fig. 2), the other is situated in the kinase domain. The hot spot mutations result in single amino acid substitutions.

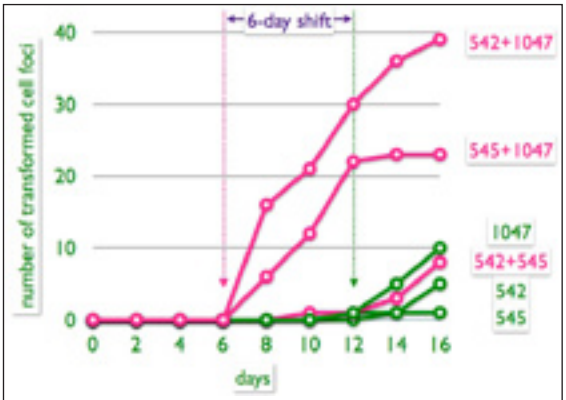

Fig. 6. Combining a mutation in the helical domain of p110 with the kinase domain mutation (545+1047 or 542+1047) in the same molecule synergistically enhances oncogenic activity. A combination of two helical domain mutations (545+542) does not increase oncogenic potency over that of single mutations.

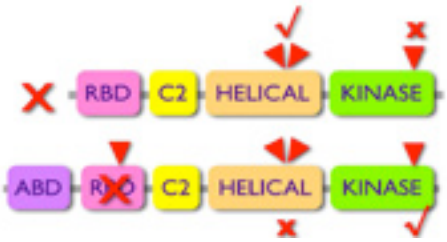

Fig. 7. The helical and kinase domain mutations in p110 are distinguished by their dependencies on interacting proteins, p85 and RAS. Eliminating the interaction with p85 through an N-terminal deletion (top) does not affect the activity of the helical domain mutations but completely inactivates the kinase domain mutation. In contrast, disruption of RAS binding through a point mutation (lower portion) in the RBD does not diminish the activity of the kinase domain mutation but inactivates the helical domain mutations.

non-defective retrovirus to act as helper and to supply essential viral functions in trans. The importance of PI3K in human cancer was revealed by the seminal discovery of tumor-specific mutations in PIK3CA, the gene encoding one of the PI3K isoforms, p110α. These mutations were found to occur mainly in two hotspots in the coding sequence, one in the helical, the other in the kinase domain of p110, suggesting that these mutations provide a selective advantage for the cell (Fig. 5). Indeed, they induce a gain of function in enzymatic and signaling activity and make p110 oncogenic in cell culture and in the animal. Molecular mechanisms for the mutation-induced gain of function are suggested by two kinds of experiments. In the first, the two hot-spot mutations are combined in the same p110 molecule. The effects are synergistic, indicating that both mechanisms are compatible in the same molecule and probably function by different molecular strategies (Fig. 6). The second type of experiment explores the interactions of these mutants with two regulatory proteins, p85 and RAS (rat sarcoma viral oncogene homolog) (Fig. 7). Disruption of the interaction with p85 inactivates the kinase domain mutation and leaves the helical domain mutation intact. Disruption of Ras binding has the opposite effect, inactivating the helical domain mutation, but leaving the kinase domain mutation active. These observations suggest two molecular mechanisms for the mutation-induced gain of function. One is represented by the helical domain mutation; it makes p110 independent of p85, presumably inducing a conformational change that is equivalent to activation by upstream signaling. The other, represented by the kinase domain mutation, results in independence from RAS binding, perhaps mimicking a conformational change that is induced by RAS in the wild-type p110. The precise nature of these activating conformational changes still needs to be worked out.

Isoforms and the regulatory subunit p85. The catalytic subunits of class I PI3K occur in four isoforms referred to as p110α, p110ß, p110γ and p110δ. They differ in structure, function and in pattern of expression. p110α and p110ß are ubiquitously expressed, p110γ and p110δ are restricted mainly to white blood cells, but can occasionally be detected in other cell types as well. The p110 isoforms show another distinguishing feature: The non-alpha isoforms are able to induce oncogenic cellular transformation when over-expressed (Fig. 8). In contrast, p110α lacks this ability. It must undergo a gain-of-function mutation to become oncogenic. Cancer-specific mutations occur only in p110α, but not in the non-alpha isoforms of p110. Yet, these non-alpha isoforms are often over-expressed in cancer. Thus, the oncogenicity of p110α is tied to mutations affecting function; that of the non-alpha isoforms results from differential expression.

Cancer-specific mutations have also been identified in the regulatory subunit p85. They are particularly common in endometrial cancer, but occur in other tumor types as well. Figure 9 shows p85 mutations identified in glioblastoma. They map to the inter-SH2 domain of p85, a domain that makes inhibitory contacts with the C2 domain of p110. The mutations disrupt these inhibitory contacts and hence lead to an activational p110. The degree of this activation is specific for a given mutation; the values as reflected in the ability of the expressed mutant to induce oncogenic cellular transformation in cell culture are spread over an order of magnitude. Presumably, this spread reflects the varying efficiency with which individual mutations disrupt the inhibitory interaction with p110. In broad outline, the p85 mutations appear to operate by the same molecular mechanisms as the helical domain mutations in p110, leading to a "disinhibition" of p110. But despite disruption of some p110 contacts, the p85 mutants still bind to p110. They probably act by increasing the enzymatic activity of p110. Binding of p85 mutants to p110α and p110β can be demonstrated by pull-down experiments (Fig. 10). This observation raises the possibility that p85 mutants work through either one or both of the p110 isoforms. This question can be investigated with the use of small molecule inhibitors that are specific for a single isoform of p110. Such experiments with small molecule inhibitors show that p85 mutant-induced oncogenic transformation and enhanced signaling are selectively sensitive to inhibitors of p110α, but are unaffected by inhibitors of p110β, p110γ or p110δ (Fig. 11). This observation could suggest a higher affinity of p85 to p110α as compared to the other isoforms; it could also be simply

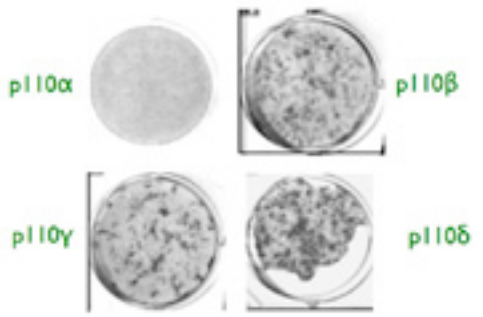

Fig. 8. Transfection of different dishes of chicken embryo fibroblast with equal amounts of DNA expressing the wild-type proteins p110β, p110γ or p110δ leads to oncogenic transformation. In contrast, transfection with DNA expressing wild-type p110α does not result in oncogenic changes.

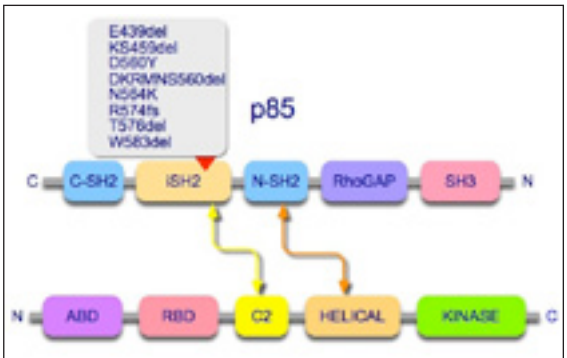

Fig. 9. Cancer-specific mutations of p85 identified in glioblastoma. All of these mutations map to the iSH2 (inter-SH2) domain. They disrupt, with differing efficiencies, the inhibitory interactions between the p85 iSH2 domain and the C2 domain of p110. Other inhibitory contacts exist between the cSH2 domain of p85 and the helical domain of p110. These contacts are disrupted by the mutations in the helical domain of p110α.

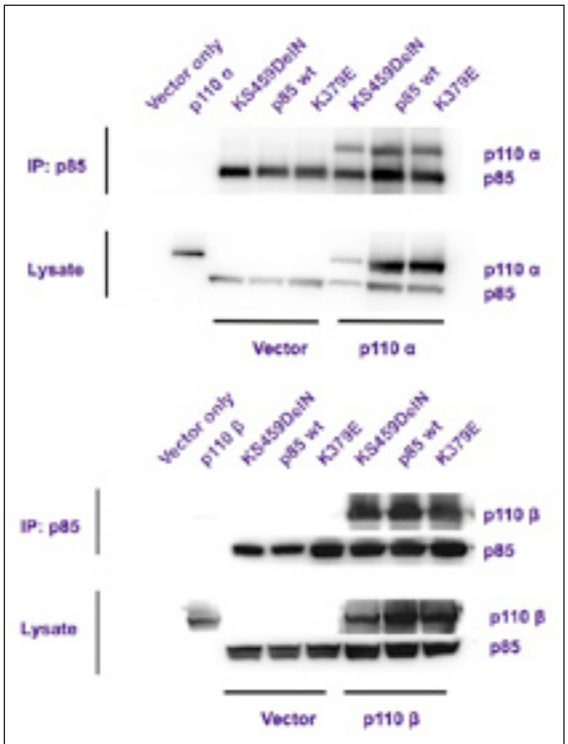

Fig. 10. "Pull-down" experiments demonstrating that immuno-precipitation of mutant p85 also precipitates p110α and p110β, hence the mutant p85 still binds to the catalytic subunit of PI3K.

reflection of the relative abundance of the isoforms in the cells used for this experiment.

Proteomics of PI3K-induced cellular transformation. In recent years, powerful methods have become available that allow comprehensive characterizations of the cellular proteome. One of these is SILAC (stable isotope labeling with amino acids in cell culture) (Fig. 12). We applied this approach to a pair of isogenic cell lines: the mouse fibroblast line 10T½ and the same cell oncogenically transformed by the cancer-specific kinase domain mutation of p110α. The mutant protein is expressed in the transformed cell line by a retroviral expression vector, generating a transgenic derivative of the original cell. We determined the relative abundance of about 5000 proteins, comparing these isogenic cells. In the transformed cells, about 100 proteins were significantly upregulated and 50 were downregulated. A gene ontology analysis identified several potential targets of STAT (signal transducer and activator of transcription) transcription factors among the most highly upregulated proteins. Western blots revealed enhanced tyrosine phosphorylation of STAT3 and of STAT6 in the transformed cells. Because of the established relationship of STAT3 to cancer, this new link between PI3K transformation and activated STAT3 was followed up. The phosphorylation of STAT3 at tyrosine 705 was reduced in the presence of inhibitors of PI3K, and expression of a dominant negative STAT3 interfered with PI3K-induced oncogenic transformation. These observations suggest that phosphorylation of STAT3 in these cells is PI3K-dependent and that STAT3 activation is required for PI3K-induced oncogenic transformation. A possible link between PI3K and STAT3 could be provided by a member of the TEC kinase family. TEC kinases are tyrosine kinases that contain an N-terminal PH (pleckstrin homology) domain. The PH domain mediates recruitment of the kinase to the product of PI3K, PIP_3, followed by activation, presumably through cross-phosphorylation of the TEC kinases themselves.

There are two ubiquitously expressed members of the TEC kinase family, TEC kinase itself and BMX (bone marrow X-linked non-receptor tyrosine kinase). Of these two, only BMX was found to be phosphorylated in the 10T½ cells and hence is a likely activator of STAT3 in these cells. Support for the involvement of TEC kinases in the activation of STAT3 comes from a small molecule TEC kinase-specific inhibitor that can be shown to

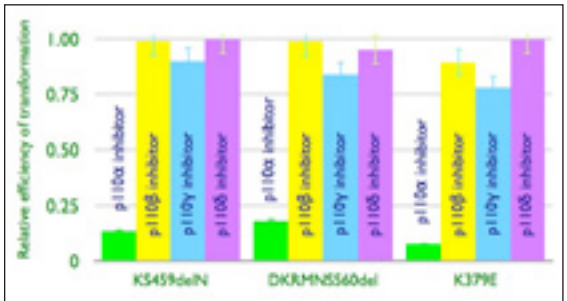

Fig. 11. Three p85 mutants are tested for their sensitivity to isoform-specific inhibitors of p110. Only inhibitors of p110a can reduce the oncogenic activity of the mutants.

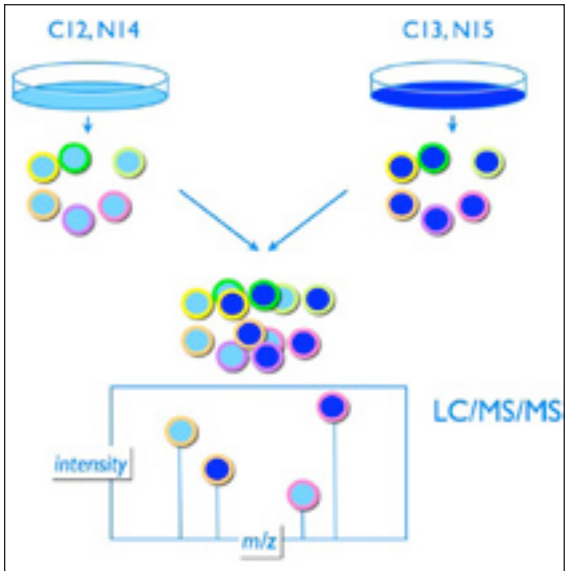

Fig. 12. In this SILAC experiments the relative abundances of numerous proteins in two isogenic cell lines are compared. The cells are 10T½ murine fibroblasts and the same cells carrying the oncogenic PI3K transgene p110a H1047R. The wild-type 10T½ cells are grown in media containing ^{12}C and ^{14}N, the H1047R carrying cells are cultivated in media containing ^{13}C and ^{15}N until a steady state of protein label with these heavy isotopes is reached. The proteins from both cell lines are then extracted; equal quantities are mixed, and the mixture is digested with trypsin. The resultant peptides resolved chromatographically and by mass spectrometry. Peptides of the same sequence but containing different isotopes of C and N can be distinguished. Their relative abundances and those of the cognate proteins are determined.

interfere with phosphorylation of STAT3 and with PI3K-induced oncogenic transformation. Collectively, the data from this SILAC comparison of 10T½ cells and the same cells transformed by a PI3K mutant reveal signaling through a TEC kinase, probably BMX, to STAT3. Activation of STAT3 is an important component of PI3K-induced oncogenic transformation (Fig. 13). In an application of this insight to a collection of human cancer cell lines, it was found that about half of these cell lines show the expected sensitivities of STAT3 phosphorylation to both inhibitors of PI3K and of TEC kinases.

Effects of a PI3K mutation on a human cell line. We have recently expanded comprehensive comparisons to a pair of isogenic human cell lines, MCF-10A and the same cell carrying a single-allele knock-in of the H1047R mutation of the gene encoding p110α, PIK3CA (Fig. 14). The knock-in was generated by Ben Ho Park of Johns Hopkins University. MCF-10A is derived from human breast epithelium. It is negative for the expression of HER2, for the estrogen receptor and the progesterone receptor ("triple negative"). The cell has two tumor suppressors silenced or deleted: CDKN2A (p14ARF) and PTPRD (protein tyrosine phosphatase). MCF10A also shows amplification of Myc. It expresses EGFR (epidermal growth factor receptor) and is dependent on EGF for continuous replication. The mutant knock-in carries a single nucleotide substitution (H1047R) in one allele of the PIK3CA gene. Exon sequencing reveals no other cancer-relevant mutations in the knock-in cells. We compared these isogenic cells by SILAC, RNAseq and miRNA profiling. These comparisons identified differentially expressed proteins and RNAs. The differential expressions of an RNA and a protein assigned to the same gene are concordant (meaning that both are upregulated or both are downregulated) in only about 50% the cases. Discordant regulation of protein and RNA are frequent. Despite these divergences, it is possible to identify broad signatures that are upregulated in the knock-in cell line. These include signatures of NFκB activity, of STAT activity and RAS activity as well as specific features of metabolism, lipid synthesis and extra-cellular matrix proteins. The extent by which the single nucleotide substitution in PIK3CA affects protein and RNA expression is surprising and so is the diversity of changes affecting cellular activities that appear not

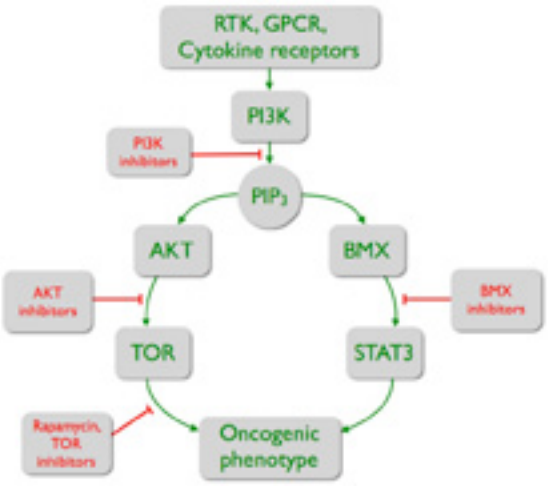

Fig. 13. PI3K is stimulated by upstream signaling that originates in RTKs or GPCRs. The oncogenic signal emanating from PI3K splits at PIP_3. One branch proceeds through AKT to TORC1, the other extends through BMX to STAT3, and both contribute to the PI3K-induced cellular oncogenic phenotype. Small molecule inhibitors for several steps of this composite signal are in clinical trial.

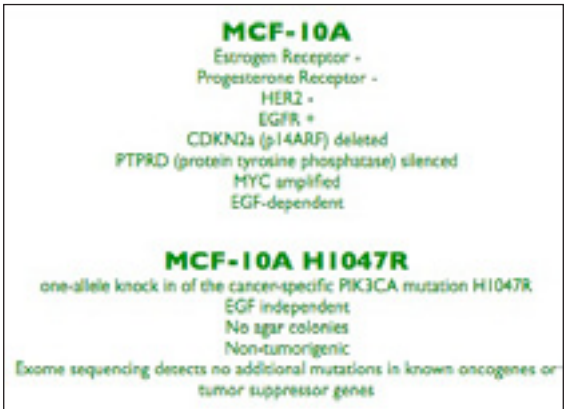

Fig. 14. Global comparisons of RNA expression profiles, of miRNA signatures and of relative protein abundances were carried out on two isogenic human cell lines. MCF10A is an immortal breast epithelial cell line, and MCF10A H1047R is the same cell with a single-allele knock in of the H1047R mutation in the gene encoding p110a, PIK3CA. The knock-in was generated by Ben Ho Park of Johns Hopkins University. The cells differ by a single nucleotide substitution in the PIK3CA gene. The knock-in differs from the wild-type by its independence from EGF.

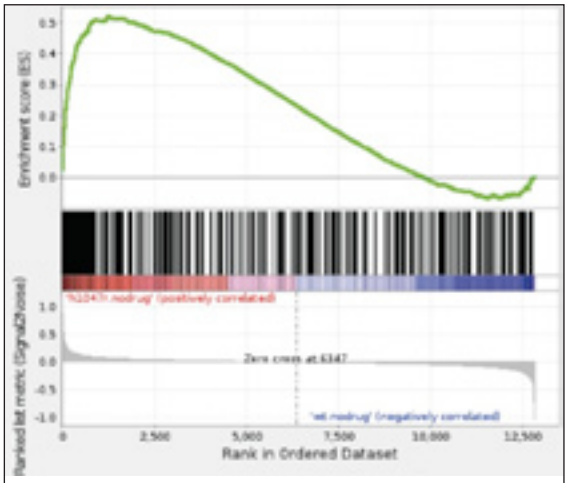

Fig. 15. Gene set enrichment analysis shows that the RNA and the protein expression profiles of the MCF10A H1047R cells closely resemble published profiles of basal (triple negative) breast cancer. A single nucleotide substitution can drive complex changes that move the expression profiles of a normal cell far toward those of an aggressive human cancer.

directly linked to PI3K. We applied gene set enrichment analysis, a technique developed by scientists of the Broad Institute, Harvard University, to the SILAC and RNAseq data from the MCF-10A and knock-in cells (Fig 15). The unexpected result of this analysis is that the single nucleotide substitution in the knock-in cell shifts protein and RNA expression profiles strongly toward the differential expression profiles characteristic of basal (triple negative) breast cancer.

Current sequencing techniques have uncovered an abundance of genetic changes in cancer cells. Even after most of these changes are discounted as insignificant bystanders, there still remain numerous mutations of potential significance for the oncogenic phenotype. This multiplicity has given rise to the suggestion that the role of individual mutations must be marginal and hence that there are no real dominant driver mutations. The fact that the 1047 mutation in p110α, a single nucleotide substitution, can induce deep changes in RNA and protein profiles and can reproduce a large proportion of the expression signature of basal breast cancer challenges this conclusion. It is clear that there are mutations, even single point mutations, that can push the cell far into the oncogenic phenotype. This is encouraging news for the development of PI3K-based therapy. PI3K can be a potent and perhaps dominant cancer driver, and shutting this force down is bound to have significant beneficial effects in the clinic.

Acknowledgments:

Research reported in this publication was supported by the National Cancer Institute under award numbers CA078230, CA153124, CA151574 and AG039716 (to PKV) as well as P30 NS057096 and RR011823 (to John R. Yates).

The work discussed here was made possible by the contributions of numerous collaborators. These include Cathy Chang, Masa Aoki, Sohye Kang, Andreas Bader, Leyna Zhao, Petra Hillmann, Minghao Su, Lynn Ueno, Jonathan Hart, Lujian Liao, Yaoyang Zhang, and John Yates.

This is manuscript 24044 of The Scripps Research Institute.

2014 Pezcoller Symposium

'Cancers Driven by Hormones'
June 19-21, 2014
Trento, Italy

The program has been formulated by

Dr. David Livingston
(Dana Farber Cancer Institute, Boston)

Dr. Myles Brown
(Dana Farber Cancer Institute, Boston)

Dr. Arul Chinnaiyan
(Michigan Centre for Translational Pathology, AnnArbor, MI)

Dr. Antonella Farsetti
(Istituto Nazionale Tumori Regina Elena IRCCS, Roma)

Dr. Massimo Loda
(Dana Farber Cancer Institute and Harvard Medical School, Boston)

Dr. Roland Schüle
(University of Freiburg Medical Centre)

With the support of Dr. Henry Mihich
(Dana Farber Cancer Institute, Boston)

Focus and Goals: Hormonal influences affect the incidence, natural history, and clinical outcomes of important and very common cancers. The molecular events that underlie these influences are increasingly apparent, and the science in this area is both fervent and incisive. Indeed, it has continued to bring about advances in clinical care that are based upon ever deeper biochemical, cell biological, physiological, and pathophysiological insights. This Symposium will explore these advances both those arising from elegant analyses of tumor biology and those of a translational nature that have emerged from them.

The final program will be available on www.pezcoller.it

Elena Favaro, Oxford University, Valentina Audrito, Turin University and Martina Chiu, Parma University, winners of the 2013 "Pezcoller Begnudelli Awards" for the best posters.

Save the date!

26th Pezcoller Symposium

June 19-21, 2014
Trento, Italy

Cancers Driven by Hormones

The Pezcoller Foundation

Journal

Six-monthly review of the
Pezcoller Foundation
Via Dordi 8 - 38122 Trento - Italy
Tel. (39) 0461 980250
Fax (39) 0461 980350
e-mail: pezcoller@pezcoller.it
www.pezcoller.it

Proprietario/editore:
Fondazione Prof. Alessio Pezcoller - Trento
n. 36 - Registro delle Persone Giuridiche
presso il Commissario del Governo
della Provincia di Trento
Redazione: Via Dordi 8 - 38122 Trento
Direttore Responsabile: Gios Bernardi

"The Pezcoller Foundation Journal"
year 23, n. 41, Semestrale ottobre 2013
Poste Italiane spa
Spedizione in abbonamento postale
D.L. 353/2003 (conv. In L. 27/02/2004 n. 46)
Art. 1, comma 2, CNS Trento
taxe percue / tassa riscossa