Metformin and Statins in PCa

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It is well known that prostate cancer is often found in inflammatory cases. It is also well known that Type 2 Diabetes and atherosclerosis are the result of inflammatory processes. Thus if one could deal with the two disorders, via therapeutics, perhaps the result would be a diminution of PCa. In a recent study by Danzig et al this effect is examined and the results are promising.

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Contents

1 INTRODUCTION

Prostate cancer has frequently been seen related to inflammatory processes. The exact connection is yet to be determined. However recent results have indicated that metformin has shown some effect on PCa and a recent paper by Danzig et al shows significant effects with metformin and statins. Both drugs have a certain antiinflammatory role, one in glucose metabolism management and the other through lipid pathways. In this paper we examine both the Danzig et al results as well and the details regarding the specific pathways involved. Specifically the drugs deal with metabolic related pathways, which is no surprise given the nature of Type 2 Diabetes. However the statin usage is not directly metabolic but may very well be so.

Shao et al state[1]:

The widely used anti-diabetic drug metformin has been shown to exert strong antineoplastic actions in numerous tumor types, including prostate cancer (PCa). In this study, we show that BI2536, a specific Plk1 inhibitor, acted synergistically with metformin in inhibiting PCa cell proliferation. Furthermore, we also provide evidence that Plk1 inhibition makes PCa cells carrying WT p53 much more sensitive to low-dose metformin treatment. Mechanistically, we found that co-treatment with BI2536 and metformin induced p53-dependent apoptosis and further activated the p53/Redd-1 pathway.

Moreover, we also show that BI2536 treatment inhibited metformin-induced glycolysis and glutamine anaplerosis, both of which are survival responses of cells against mitochondrial poisons. Finally, we confirmed the cell-based observations using both cultured cell-derived and patient-derived xenograft studies. Collectively, our findings support another promising therapeutic strategy by combining two well tolerated drugs against PCa proliferation and the progression of androgen-dependent PCa to the castration-resistant stage.

For example in the work of Margel et al they note:

By using fractional polynomials, we verified that the association between cumulative metformin use after PC diagnosis and PC specific mortality is linear. Onmultivariable analysis, for each additional 6 months of metformin use after PC diagnosis, there was a 24% reduction in PC-specific mortality (adjusted HR [aHR], 0.76; 95% CI, 0.64 to 0.89). Increasing durations of cumulative use of all other antidiabetic medications was not associated with PC-specific mortality.

In a similar manner in a study with statins Allott et al noted[2]:

In this retrospective cohort of men undergoing RP, post-RP statin use was significantly associated with reduced risk of BCR. Whether the association between post-RP statin use and

[1] http://www.jbc.org/content/290/4/2024.abstract

[2] http://onlinelibrary.wiley.com/doi/10.1111/bju.12720/abstract

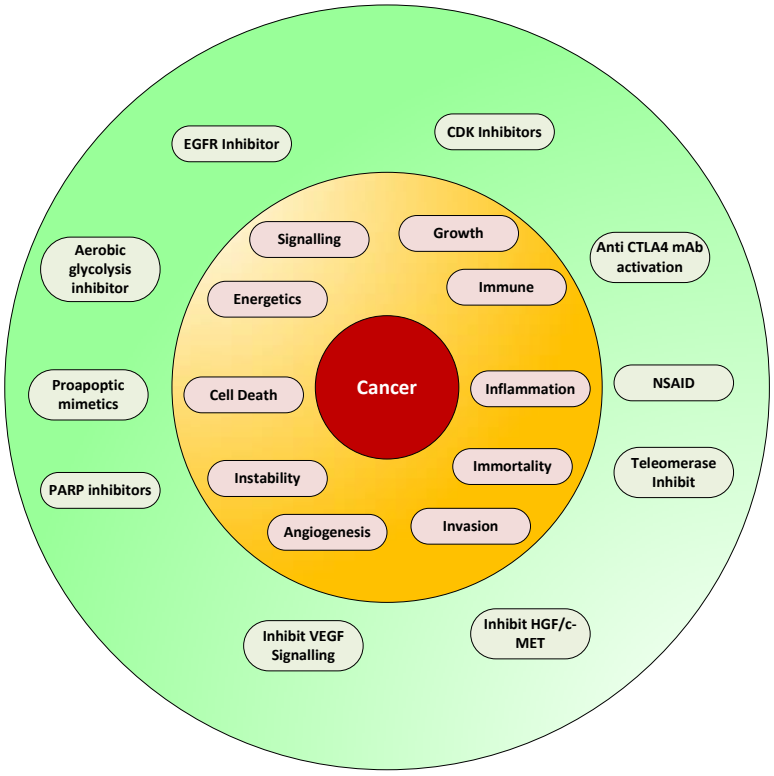
BCR differs by race requires further study. Given these findings, coupled with other studies suggesting that statins may reduce risk of advanced prostate cancer, randomized controlled trials are warranted to formally test the hypothesis that statins slow prostate cancer progression.

Thus it would be reasonable to try an analysis with metformin and a statin combined. It is this study that we have focused upon as a vehicle to explore the effects on prostate cells using drugs that have effects on processes which are fundamentally inflammatory; excess blood glucose and excess blood lipids. To do this we sue the most recent paper of Danzig et al where they state:

The combination of statins and metformin in men undergoing RP for prostate cancer (PCa) may be associated with a lower BCR risk than would be predicted based on the independent effects of both medications. A synergism between these two agents is biologically plausible based on our current understanding of their diverse molecular pathways of action. The results of future clinical trials involving the use of either medication in men with PCa should be carefully assessed for confirmatory evidence of such a relationship.

Thus there may very well be a beneficial result of such an approach. We briefly examine this and the details beneath in terms of the cellular pathway dynamics.

We describe graphically below many of the elements which we consider when examining cancer and its causes. This paper is less a direct examination of causes than recognition of the effects of well understood medications on pathways and putatively their impact on the cancer process.



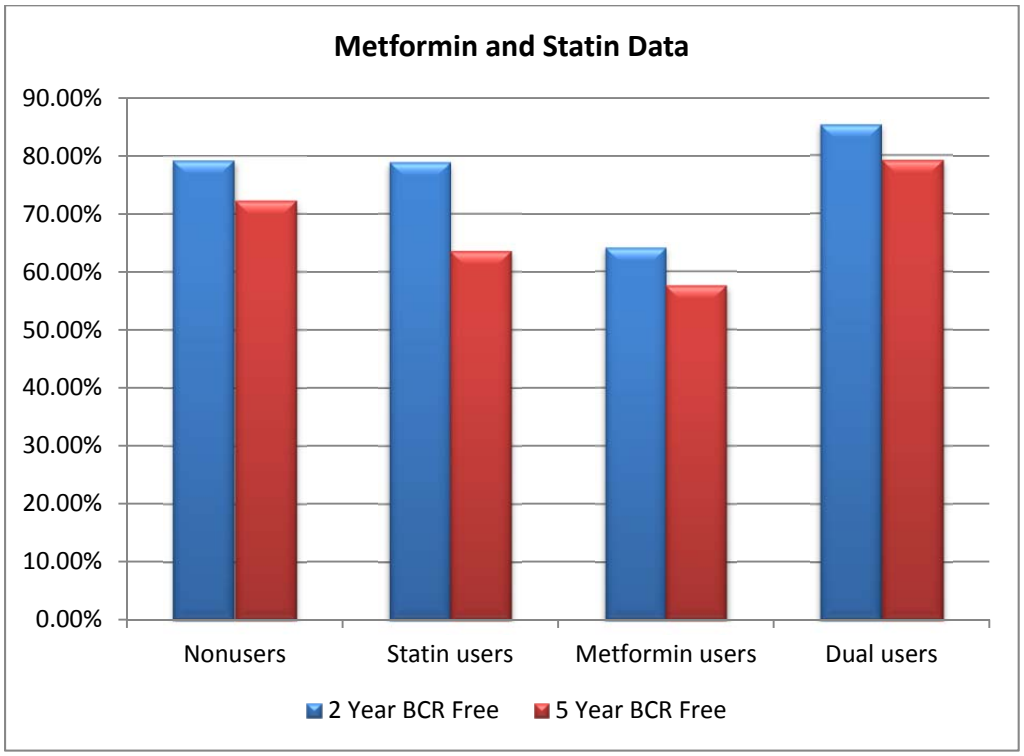
In this analysis we utilize the Danzig et al paper and examinie in some details the functions of the specific drugs and their pathway characteristics. We specifically focus on metabolic pathway

elements such as mTOR, AMPK, and how these are influencing a pathogenic characteristic leading to PCa.

2 RESULTS

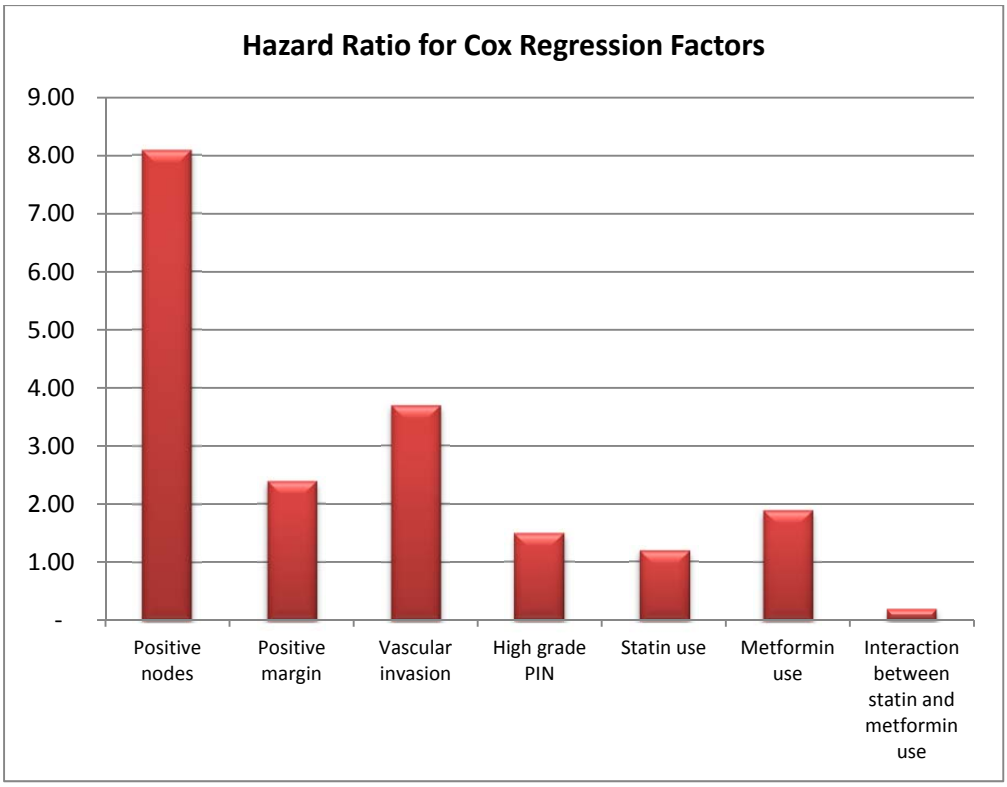
Our focus is on the results from the Danzig et al paper. It demonstrates a synergism between metformin and statins in reducing mortality from both HGPIN and PCa. The issue of concern is; just how do these two medications function and what if anything can be generalized from this observation? It is well known that statins have an ameliorative effect on certain cancers and it is also well known that cancers can be initiated and exacerbated by inflammatory processes such as Type 2 Diabetes. We examine some of the basic observations presented in the paper and then proceed to examine the details of the pathway controls.

From the Dantzig paper we have the following survival across the four groups:

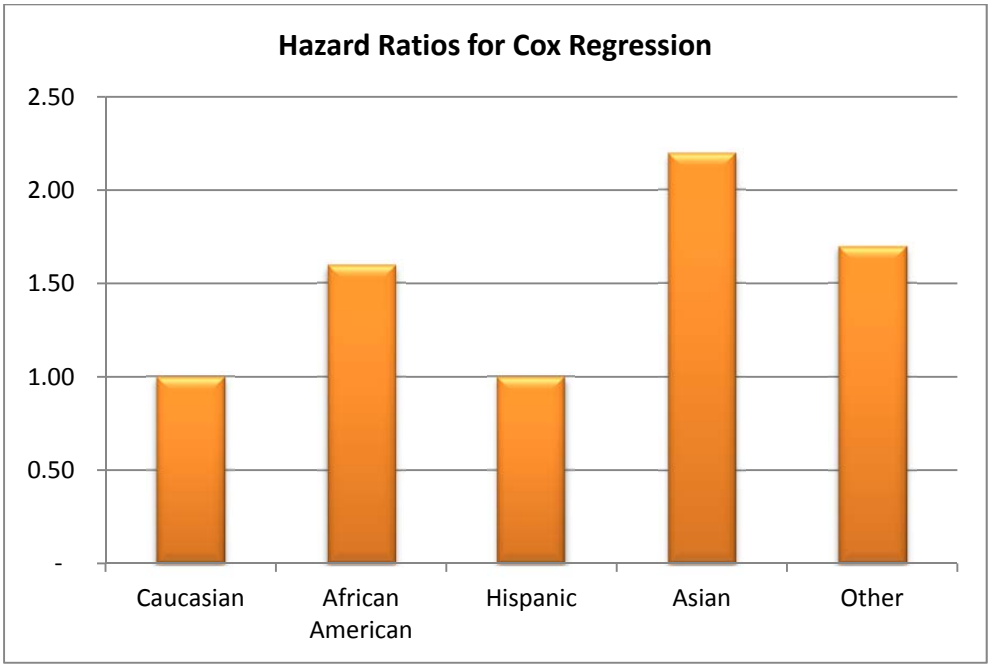


Note the alleged improvement. Also presented in the paper are Hazard Ratios. We summarize three key ones below.

First, we summarize the results of Hazard Ratios on several key factors in the initial stages of presentation. These are all related to biochemical recurrence, BCR.

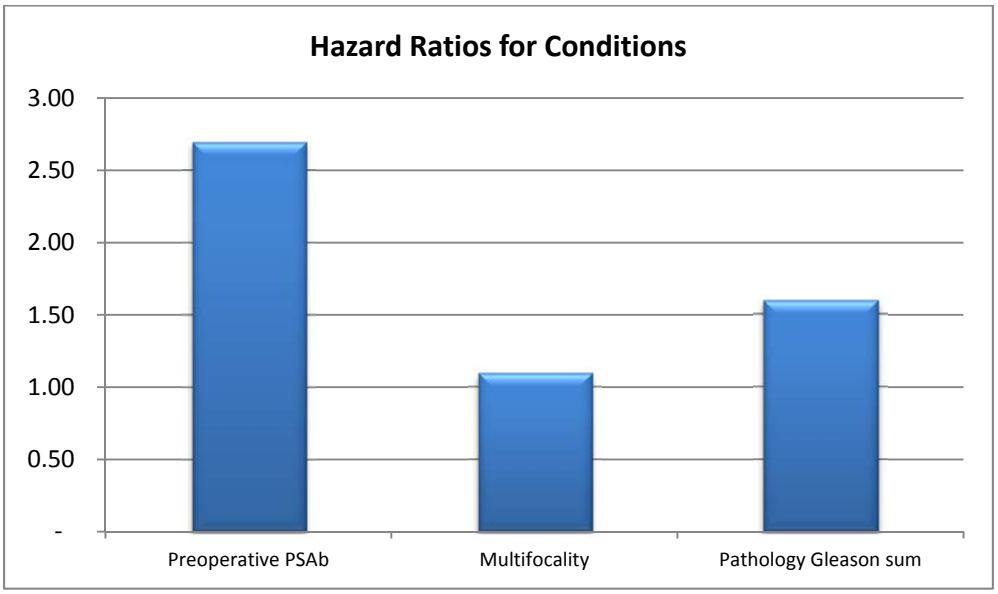


Second the Hazard Ratios for race are presented. Surprisingly Asia is higher than African American.



Third, below is the Hazard Ratio summary for conditions of the lesion. What is interesting is the importance of pre-operative PSA levels. Perhaps this is a marker for reflecting on the importance

of continuing to measure PSAs since the higher it is pre-operatively the greater the chance of post-operative recurrence.



As Danzig et al conclude:

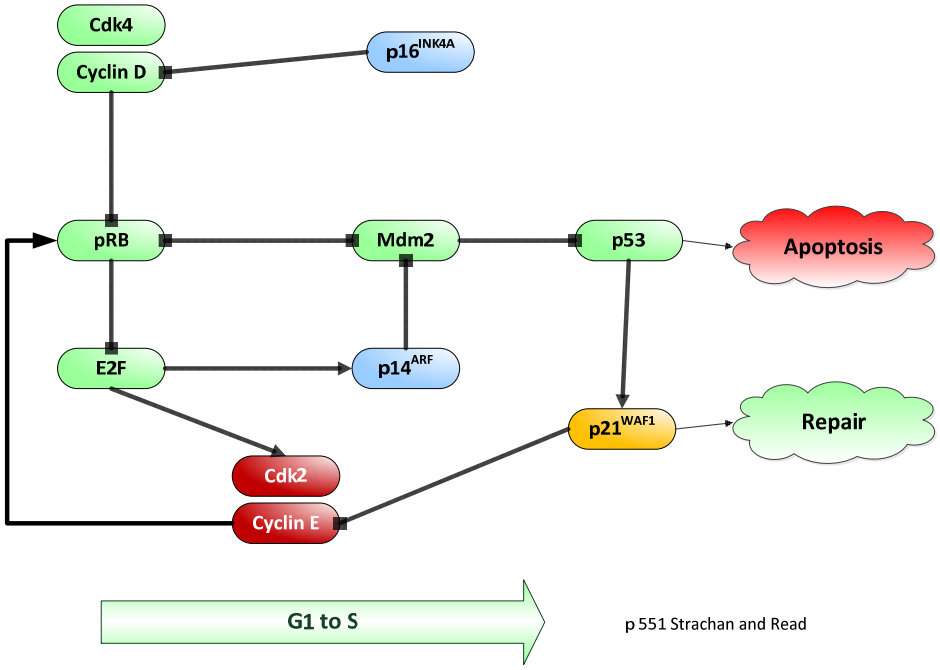
In conclusion, we found that the combination of statins and metformin in men undergoing RP for PCa may be associated with a lower BCR risk than would be predicted based on the independent effects of both medications. A synergism between these two agents is biologically plausible based on our current understanding of their diverse molecular pathways of action. The results of future clinical trials involving the use of either medication in men with PCa should be carefully assessed for confirmatory evidence of such a relationship. Finally, continued research into the molecular mechanisms by which these drugs affect cancer behavior will be highly instructive.

Thus the study presents some significant additional insight into pathways via the use of these medications. We thus start with pathways and then consider the effects of the medications.

3 SOME CONTROL POINTS

We consider a brief review of some of the metabolic pathways in a cell whose loss of control are frequently aligned with a malignant growth. The factors we look at are those that lead to cell proliferation via cell cycle loss of control and loss of normal cell apoptosis as well as loss of repair capability.

Let us consider now the cell control mechanism of p53 as a prime example, we show this below:



The above demonstrates some of the key principles we will discuss. p53 is a key gene product whose control of cell growth and reproduction is essential and any blockage of its function can result in a malignant growth. This has been understood now for several decades. There are also other control gene products such as p21, p16, and p14 as well as the MDM2 genes products that all play a key role.

Now metabolic factors in a cells environment place stress upon a cell that can result in loss of control as shown above. One metabolic or environmental factor is inflammation, others such as excess glucose or loss of glucose control is another. We examine the latter here.

For example, regulating p53 expression is known to be a major goal. Loss of that regulation is a major concern. One of the major players in that role is AMPK, AMP kinase. AMPK is a metabolic regulatory gene product that on the one hand manages cell energy control and on the other hand can control p53. Thus controlling this element is essential.

This then leads us to other gene products such as mTOR and essential metabolic gene product as well as LKB1.

3.1 AMPK PATHWAY

Cell metabolism is the process whereby a cell uses energy that is made available to it to maintain normal processes and to grow and reproduce as may be required. Normal metabolic processes in a cell allow for the control of all of the elements in a balanced manner. Excess glucose as seen in Type 2 Diabetes can result in quasi-inflammatory states and loss of homeostasis.

Let us focus briefly upon AMPK, AMP kinase, as an initial point to understand the intra-cellular metabolic processes. AMPK is a key control element in many intracellular pathways[3].

From the paper by Mihaylova and Shaw we have[4]:

One of the central regulators of cellular and organismal metabolism in eukaryotes is AMP-activated protein kinase (AMPK), which is activated when intracellular ATP production decreases.

AMPK has critical roles in regulating growth and reprogramming metabolism, and has recently been connected to cellular processes such as autophagy and cell polarity. Here we review a number of recent breakthroughs in the mechanistic understanding of AMPK function, focusing on a number of newly identified downstream effectors of AMPK.

From the work of Shackelford and Shaw we have[5]:

In the past decade, studies of the human tumour suppressor LKB1 have uncovered a novel signalling pathway that links cell metabolism to growth control and cell polarity.

LKB1 encodes a serine–threonine kinase that directly phosphorylates and activates AMPK, a central metabolic sensor. AMPK regulates lipid, cholesterol and glucose metabolism in specialized metabolic tissues, such as liver, muscle and adipose tissue. This function has made AMPK a key therapeutic target in patients with diabetes.

The connection of AMPK with several tumour suppressors suggests that therapeutic manipulation of this pathway using established diabetes drugs warrants further investigation in patients with cancer.

In particular Shackelford and Shaw demonstrate the impact of Metformin on this pathway.

As Mendelsohn et al state:

[3] http://www.cellsignal.com/contents/science-pathway-research-cellular-metabolism/ampk-signaling-pathway/pathways-ampk This is a useful pathway description worth examining in detail.

[4] http://www.nature.com/ncb/journal/v13/n9/full/ncb2329.html

[5] http://www.nature.com/nrc/journal/v9/n8/full/nrc2676.html

While growth factor–stimulated signaling cascades promote cell growth under favorable conditions, cells have sophisticated nutrient sensing systems that serve to block growth when the internal energy supply is limiting. These regulators ensure that, during periods of intracellular nutrient depletion, metabolites are redirected from anabolic pathways and instead used to fuel catabolic pathways that will provide the energy required to survive the period of nutrient limitation. The AMP-activated protein kinase (AMPK) plays a major role coordinating cellular energy status with appropriate metabolic responses.

AMPK directly senses cellular energy levels in the form of the AMP/ATP ratio. Falling energy levels increase the cellular AMP/ATP ratio, priming AMPK for activation by the liver kinase B1 (LKB1). AMPK phosphorylates multiple targets with the cumulative effect of blocking anabolic reactions and stimulating energy-generating catabolic pathways.

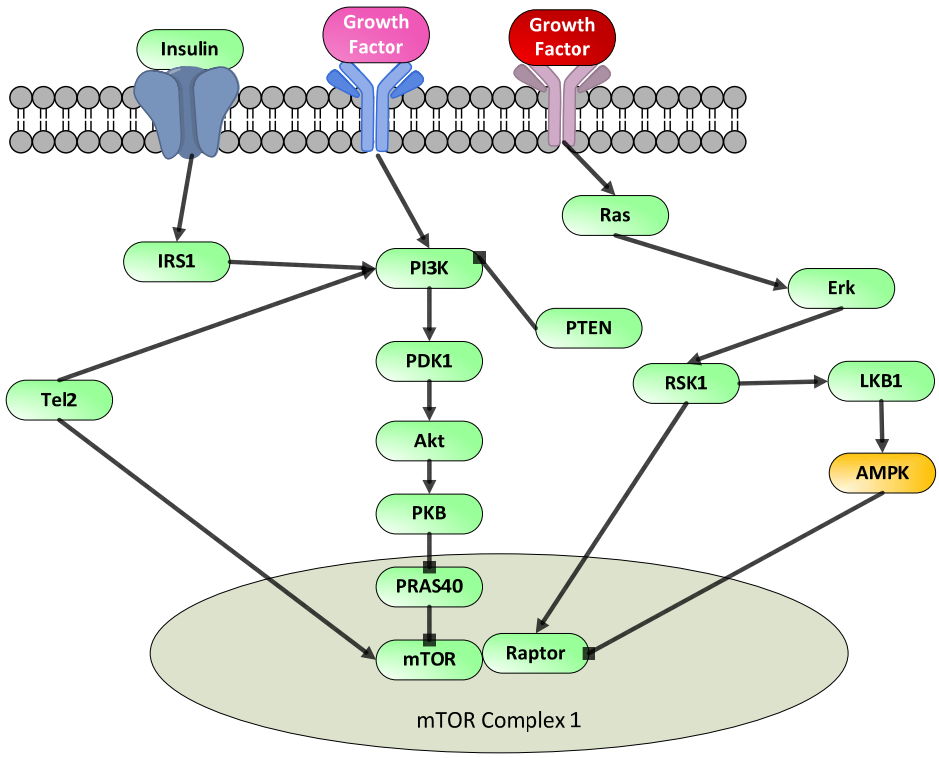
For example, AMPK phosphorylates and inhibits acetyl-CoA carboxylase (ACC), with the dual effect of blocking fatty acid synthesis and activating fatty acid oxidation. AMPK also directly inhibits cell growth, both by inducing a p53-dependent cell cycle arrest and by blocking mTOR activity at multiple levels. Through these diverse activities, AMPK functions as a metabolic checkpoint, ensuring that cell growth is halted until bioenergetic conditions are favorable for growth.

AMPK is a powerful regulator of cell dynamics. It senses and manages energy via the ATP control cycle. Its impact on p53 which we have discussed earlier is also a major factor which may lead to cell oncogenesis. Thus examining how AMPK reacts to excess glucose and how it can be reset is a key observation.

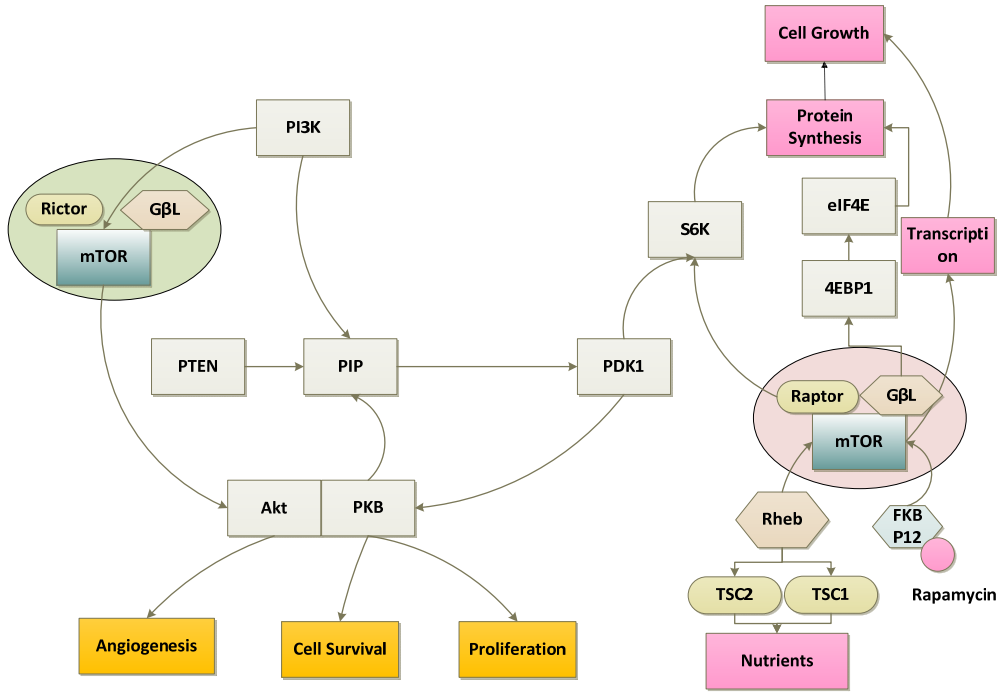
3.2 mTOR Elements

mTOR is a control protein that in involved in metabolic related pathways. mTOR, the mammalian target of rapamycin, is a gene product (1p36.2) is a protein which acts in a critical manner in interconnecting the genetic circuits in mammals, and especially man. It fundamentally controls glucose transport and protein synthesis. The pathway depicted below is a modification of the graphic from Weinberg (p 785) which shows mTOR in its two modes, one with Raptor assisting and one with Rictor. The Rictor/mTOR mode activates the Akt pathway via the placement of a phosphate and this manages the protein synthesis portion. The inclusion of rapamycin will block the Raptor/mTOR path and reduce the protein synthesis and cell growth portion. The inhibitory effect on Akt/PKB by rapamycin is assumed to be the main factor in its anti-cancer effects.

We depict the mTOR C1 pathway below:



The following chart presents a more complex version of the mTOR C1 pathway (Raptor). This allows us to best understand the complex interactions. The mTOR C1 and C2 pathways are depicted in the combined chart below:



Looking at the complexity of the mTOR pathway it presents an interesting one for addressing PCa. Kinkaide et al (2008) indicate:

Among the major signaling networks that have been implicated in advanced prostate cancer are the AKT/mammalian target of rapamycin (AKT/mTOR) and MAPK pathways. Indeed, deregulated expression and/or mutations of the phosphate and tensin homolog tumor suppressor gene (PTEN) occur with high frequency in prostate cancer, leading to aberrant activation of AKT kinase activity as well as its downstream effectors, including the mTOR signaling pathway. In addition, many prostate tumors display deregulated growth factor signaling, which may result in activation of MAPK kinase 1 (MEK) kinase and ultimately ERK MAP.

Notably, previous studies have demonstrated that the AKT/mTOR and MAPK signaling pathways are alternatively and/ or coordinately expressed in advanced prostate cancer and function cooperatively to promote tumor growth and the emergence of hormone- refractory disease. These observations formed the basis for our hypothesis that targeting these signaling pathways combinatorially may be effective for inhibiting tumorigenicity and androgen independence in prostate cancer.

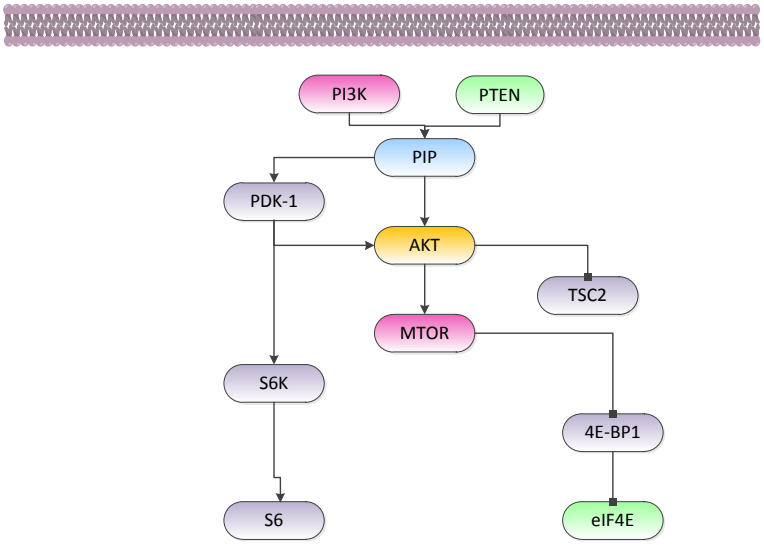
Kinkaide et al also demonstrate the creation of HGPIN via their work. This represents another pathway of HGPIN to PCa.

LoPiccolo et al state:

The PI3K/Akt/mTOR pathway is a prototypic survival pathway that is constitutively activated in many types of cancer. Mechanisms for pathway activation include loss of tumor suppressor PTEN function, amplification or mutation of PI3K, amplification or mutation of Akt, activation of growth factor receptors, and exposure to carcinogens. Once activated, signaling through Akt can be propagated to a diverse array of substrates, including mTOR, a key regulator of protein translation. This pathway is an attractive therapeutic target in cancer because it serves as a convergence point for many growth stimuli, and through its downstream substrates, controls cellular processes that contribute to the initiation and maintenance of cancer.

Moreover, activation of the Akt/mTOR pathway confers resistance to many types of cancer therapy, and is a poor prognostic factor for many types of cancers. This review will provide an update on the clinical progress of various agents that target the pathway, such as the Akt inhibitors perifosine and PX-866 and mTOR inhibitors (rapamycin, CCI-779, RAD-001) and discuss strategies to combine these pathway inhibitors with conventional chemotherapy, radiotherapy, as well as newer targeted agents. We (show) how the complex regulation of the PI3K/Akt/mTOR pathway poses practical issues concerning the design of clinical trials, potential toxicities and criteria for patient selection.

LoPiccolo et al show the more simplified pathway as follows:

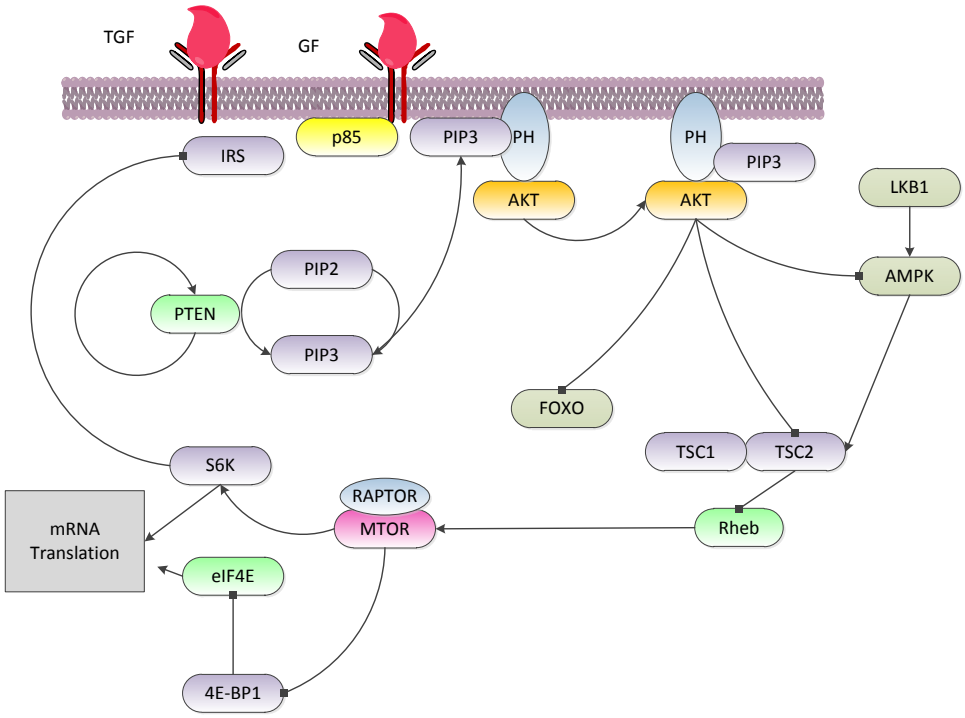


As we have shown with the more complex Weinberg model, here mTOR and PTEN play a strong role in the overall control. The authors show the points of possible control. The complexity of the pathways will be a challenge. It is less an issue of size complexity than a feedback and instability complexity. Nelson et al (2007) have demonstrated similar results as well.

Other researchers have also posited other simple models. We demonstrated the one by Hay as has been stated:

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 Akt mTOR interplay for cancer progression and therapy.



The Baldo et al model is quite similar to the Weinberg model shown initially. It clearly demonstrates the overall controlling influence of mTOR. As Baldo et al state:

There is a great body of evidence supporting consideration of the mTOR signaling system as an important network in cell regulation, differentiation and survival. mTOR is a sensor of mitogen, energy and nutritional levels, acting as a "switch" for cell-cycle progression from phase G1 to phase S.

The antibiotic Rapamycin, a potent mTOR inhibitor, has been known to the National Cancer Institute and recognized for its potential anticancer properties since the 1970s. The observation that cell lines from different cancer types exposed to low doses of Rapamycin underwent cell-cycle arrest in phase G1, provided the basis for considering mTOR as a target for cancer therapy.

Development of mTOR inhibitor compounds has proceeded empirically due to the lack of understanding of the precise molecular targets and the required dose of the new compounds . The development of Rapamycin analogs ("Rapalogs"), but also of other, structurally different, mTOR inhibitors, was directed at the selection of specific cancer type sensitivity and an optimization of pharmaceutical forms.

To give an example, Temsirolimus revealed clinical responses in patients with renal cell carcinoma in advanced stage. Temsirolimus was approved by the FDA on May 2007 for this therapeutic use and is being investigated in clinical trials for other cancer types (breast cancer, lymphoma, renal cancer, glioblastoma); significantly there are a considerable number of clinical studies involving mTOR inhibitors currently active worldwide…

The mTOR pathway controls cell size and cellular proliferation*.…nutrient metabolism, mRNA translation and cell survival control. Disruption of TOR leads to early embryonic death in flies and mammalian cells, indicating mTOR plays an important role in regulating cell survival. … deregulation of several mTOR components leads to modified cell proliferation patterns and, on the other, that many mTOR components are deregulated in several human cancers.*

*… **Therefore, inhibition of mTOR leads to slowing or arrest of cells in the G1 phase**. Translational control may have an important role in the balance of cell survival and death, and hence for apoptosis. Importantly, components of mTOR are deregulated in some human cancers, for example, breast and colon. Alteration of PI3-K/Akt is frequently observed in head and neck cancer .*

PTEN, a phosphatase that acts on PIP3 to convert it to PIP2, normally regulates the mTOR pathway negatively, and shows decreased activity in some tumors. A strong relation seems to exist between the sensitivity to the effect of Rapamycin and PTEN loss or deregulation. PTEN is frequently mutated in several cancers and in cancer-like syndromes like Cowden and Proteus syndromes…

Loss of PTEN function can occur in 26-80% of endometrial carcinomas, …recent studies of human prostate cancer have shown that loss of PTEN is strongly associated with more aggressive cancers. The relationship between PTEN status and sensitivity to rapalogs has been questioned by several investigators. Some attention has recently been dedicated to the role of the mTORC2 complex in the mTOR pathway.

In fact this complex, believed until recently to be completely insensitive to the effect of Rapamycin, after long-term exposure to Rapamycin is able to prevent mTOR-mediated Akt phosphorylation and the activation of the mTOR pathway. Another component, the TSC1/TSC2 complex located upstream of mTOR, is predicted to integrate signals derived from nutrients, cellular energy status and hypoxia into a common growth regulatory signal to the mTORC1 complex.

As Easton and Houghton state:

Proteins regulating the mammalian target of rapamycin (mTOR), as well as some of the targets of the mTOR kinase, are overexpressed or mutated in cancer. Rapamycin, the naturally occurring inhibitor of mTOR, along with a number of recently developed rapamycin analogs (rapalogs) consisting of synthetically derived compounds containing minor chemical modifications to the parent structure, inhibit the growth of cell lines derived from multiple tumor types in vitro, and tumor models in vivo.

Results from clinical trials indicate that the rapalogs may be useful for the treatment of subsets of certain types of cancer. The sporadic responses from the initial clinical trials, based on the hypothesis of general translation inhibition of cancer cells are now beginning to be understood owing to a more complete understanding of the dynamics of mTOR regulation and the function of mTOR in the tumor microenvironment. This review will summarize the preclinical and clinical data and recent discoveries of the function of mTOR in cancer and growth regulation.

The other observation here is that we often find multiple characterizations of the pathways. Namely there is no canonical form, and often a pathway is depicted to demonstrate a specific protein function. Thus we may see an emphasis on one set of proteins while others are neglected. As much as we currently attempt to unify this process we are left somewhat adrift in model development at this stage. This can be exemplified by now looking at the next section on LKB1. There we show its control over PTEN whereas in an earlier model we have it controlling AMPK. In reality there are multiple links as we have discussed. The literature can be even more confusing on this issue as well.

As Mendelsohn et al state:

It is now widely accepted that mTORC1 positively controls an array of cellular processes critical for growth, including protein synthesis, ribosome biogenesis, and metabolism, and negatively influences catabolic processes such as autophagy—all of which have roles in cancer pathogenesis. Elucidating the key downstream targets of mTORC1 driving these events is an intense area of research.

Originally, much of the study of mTOR relied on experiments in which rapamycin was used acutely to inhibit mTOR (which we now know was mTORC1) in cultured cells. This led to extensive characterization of the best known mTORC1 substrates eiF-4E-binding protein 1(4E-BP1) and S6 kinase 1 (S6K1), both of which regulate protein synthesis.3 In the unphosphorylated state, 4E-BP1 binds and inhibits the cap-binding protein and translational regulator eIF4E. When phosphorylated by mTOR, 4E-BP1 is relieved of its inhibitory duty, promoting eIF4E interaction with the eIF4F complex and the translation of capped nuclear transcribed mRNA.

Following co-regulatory phosphorylation by mTORC1 and another kinase called phosphatidylinositol 3-dependent kinase 1 (PDK1), S6K1 positively affects mRNA synthesis at multiple steps including initiation and elongation by phosphorylating several translational regulators. Although the preponderance of evidence indicates that S6K1 and 4E-BP1 are directly phosphorylated by mTOR, an unidentified phosphatase activity may also be involved in their regulation. For example, the rapamycin-sensitive phosphorylation site on S6K1 is rapidly dephosphorylated (i.e., within minutes) of exposure to the drug.

They continue:

Conditions that inhibit growth, such as decreased energy, low oxygen, and insufficient nutrients, are associated with the harsh microenvironment of poorly vascularized tumor. The ability of cancer cells to overcome these adverse conditions would promote tumor growth, putting the desensitization of mTORC1 signaling in the spotlight as a potential mechanism cancer cells could exploit to enhance their viability. Whether mutations in the amino acid– and glucose-sensing pathway that activates mTORC1 exist in cancer is not known. Mutations in the growth factor inputs to mTORC1 are prominent in cancer.

For example, mutations causing loss of PTEN function or oncogenic activation of PI3K or AKT are associated with many aggressive human cancers (Table 12-1).17-20 The findings that AKT

promotes mTORC1 activity through TSC and PRAS40 suggest that cancers with elevated PI3K-AKT signaling may in part thrive because of an mTORC1-driven growth advantage. Activation of PI3K-AKT signaling also facilitates nutrient uptake by cells, which indirectly contributes to mTORC1 activity by localizing mTORC1 to lysosomes.

Therefore, understanding the contribution and relevance of mTORC1 signaling in the progression of cancers with aberrant PI3K-AKT signaling is an important area of research.

3.3 LKB1

LKB1 has been demonstrated to be the underlying control element in Peutz-Jeghers syndrome, a proliferative melanocytic genetically dominant disorder. It controls certain pathways and as a result can be considered as a candidate in the development and progression of melanoma. Generally LKB1 is a gene whose protein stabilizes the growth and location of melanocytes. Understanding its impact in Peutz-Jeghers allows one to examine what happens when its function is suppressed in melanoma. Albeit not an initiator in the process, its aberration in a melanocyte argues for movement and loss of control.

In a recent paper by Liu et al the authors examine this premise and conclude that loss of LKB1 is significant especially in metastatic evolution. As Liu et al state:

Germline mutations in LKB1 (STK11) are associated with the Peutz-Jeghers syndrome (PJS), which includes aberrant mucocutaneous pigmentation, and somatic LKB1 mutations occur in 10% of cutaneous melanoma. By somatically inactivating Lkb1 with K-Ras activation (±p53 loss) in murine melanocytes, we observed variably pigmented and highly metastatic melanoma with 100% penetrance. LKB1 deficiency resulted in increased phosphorylation of the SRC family kinase (SFK) YES, increased expression of WNT target genes, and expansion of a $CD24^+$ cell population, which showed increased metastatic behavior in vitro and in vivo relative to isogenic $CD24^-$ cells. ***These results suggest that LKB1 inactivation in the context of RAS activation facilitates metastasis by inducing an SFK-dependent expansion of a prometastatic, $CD24^+$ tumor subpopulation.***

Earlier work by Zheng et al noted:

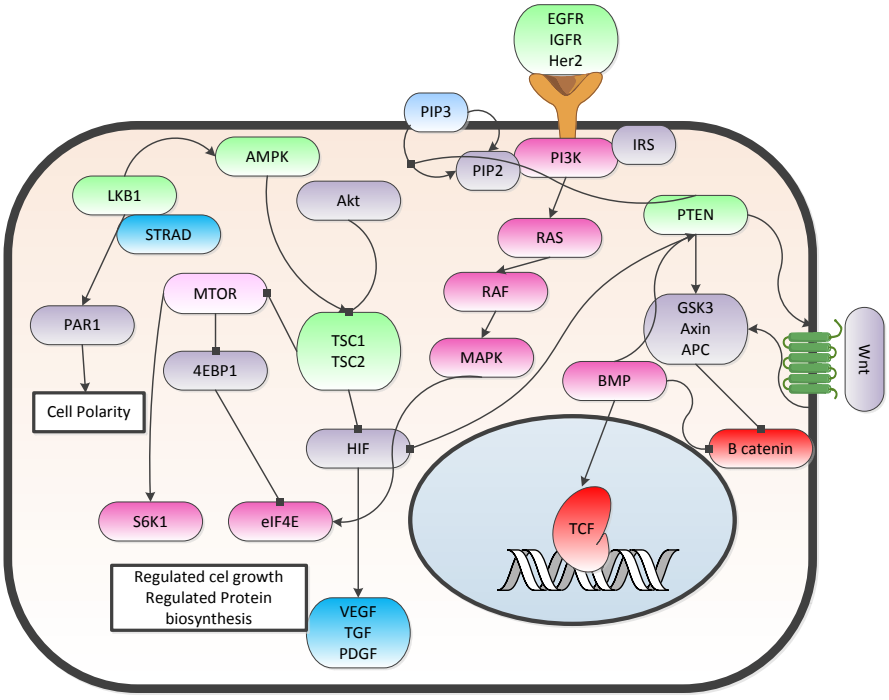
The LKB1-AMPK signaling pathway serves as a critical cellular sensor coupling energy homeostasis to cell growth, proliferation, and survival. However, how tumor cells suppress this signaling pathway to gain growth advantage under conditions of energy stress is largely unknown.

Here, we show that AMPK activation is suppressed in melanoma cells with the B-RAF V600E mutation and that downregulation of B-RAF signaling activates AMPK. We find that in these cells LKB1 is phosphorylated by ERK and Rsk, two kinases downstream of B-RAF, and that this phosphorylation compromises the ability of LKB1 to bind and activate AMPK. Furthermore, expression of a phosphorylation-deficient mutant of LKB1 allows activation of AMPK and inhibits melanoma cell proliferation and anchorage-independent cell growth.

Our findings provide a molecular linkage between the LKB1-AMPK and the RAF-MEK-ERK pathways and suggest that suppression of LKB1 function by B-RAF V600E plays an important role in B-RAF V600E-driven tumorigenesis.

Thus Zheng et al putatively identified these two pathways as sources for melanoma development. Liu et al appear to have extended this to metastasis.

Now in a paper by Bauer and Stratakis the authors provide an excellent overview of the controlling pathways. We provide a revised version of their pathway controls in a normal melanocyte below. This provides a description of the normal homeostatic pathways within a melanocyte.

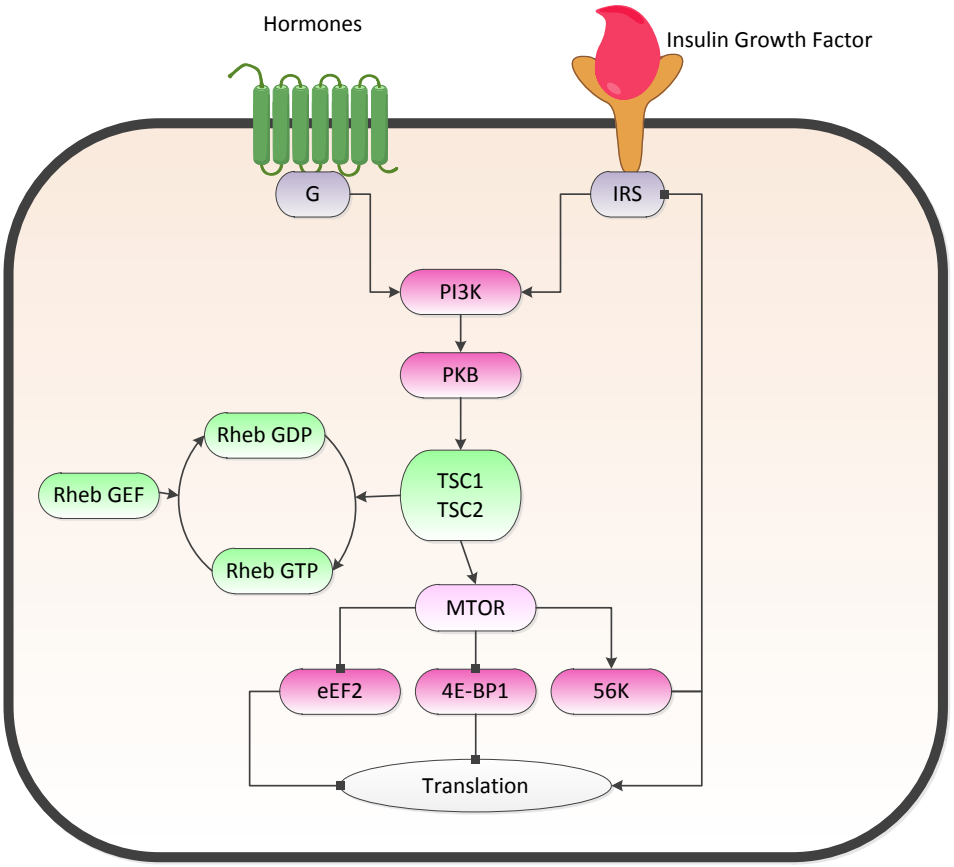


From: Bauer and Stratakis

The LKB1 gene, also called STK11, which encodes a member of the serine/threonine kinase, regulates cell polarity and functions as a tumour suppressor. This is clearly demonstrated in the above. Now recall that mTOR is a protein kinase and is a key regulator of cell growth[6]. mTOR stimulates mRNA translation thus facilitating the conversion into proteins. mTOR also facilitates the formation of ribosomes which as an important condition of cell growth under specific physiological conditions. Through the effects of mTOR on the ribosome machinery it becomes a significant factor in increasing translational activity in a cell.

We demonstrate graphically how mTOR function in some detail below:

[6] See Marks et al pp 335-345.



As Marks et al state regarding the above flow we have (p 337):

Activation and effects of the mTOR protein kinase *By inactivating the GAP TSC2 of the small G-protein Rheb, extracellular signals stimulating the PI3K-PKB signaling cascade prompt Rheb to activate mTOR. mTOR enhances the activity of the protein kinase S6K and represses 4E-BP1 and eEF2 activities, resulting in an increased rate of translation (whether 4E-BP1 and eEF2 kinase are phosphorylated directly by mTOR, as shown here, or by S6K or by both kinases is not entirely clear).*

mTOR may also be directly phosphorylated and activated by PKB.

A stimulatory effect resembling that of PKB has the MAP kinase ERK connecting mTOR signaling with mitogenesis (not shown). mTOR is also activated by nutrients such as amino acids and sugars along an ill-defined pathway that seems to include a class III PI3K.

The red dotted line (we use squared ends as compared to arrow ends) shows the negative feedback of insulin signaling: S6K phosphorylates and inactivates the insulin-specific docking protein IRS. This effect is augmented by overnutrition (leading to increased insulin release) and provides one of the causes of diabetes. Also shown is the activation of the Rheb-GAP TSC2 by 5'-AMP-dependent protein kinase (AMPK) that results in an inhibition of mTOR signaling and protein synthesis and protects the cell in situations of energy deficiency.

Now Liu et al state regarding this pathway model:

Two independent pathways appear to be critically important in regulating cell growth in response to nutrient supply and mitogenic stimulation:

(i) the PKA/PRKAR1A-LKB1 tumour suppressor protein pathway, acting via AMPK, and

(ii) the PI3K/AKT pathway.

Recent evidence suggests that the tumour suppressor gene complex, TSC1/TSC2, orchestrates the signal from both pathways to the downstream target, mTOR, which in turn regulates the ribosomal protein S6 and 4EBP-1, a repressor of the translational initiation factor eIF4E. In this model, at times of nutrient stress LKB1/AMPK activation of the TSC1/TSC2 complex results in inhibition of mTOR and a decrease in protein synthesis.

Under stimulation of mitogenic pathways, PI3K phosphorylates PIP2 to PIP3 resulting in recruitment of AKT to the membrane where it is activated by PDK1. Activated AKT inhibits the TSC1/TSC2 tumour suppressor complex leading to increased mTOR activity. In the later pathway, PTEN antagonises PIP3 action through dephosphorylation, and thus provides an ''off'' switch for regulating mitogenic pathway induced cellular growth and proliferation.

Cross talk of several other pathways appears to play important regulatory roles in the lentiginoses syndromes to include the Ras/MAPK pathway in the regulation of translation, the LKB1 pathway in cellular polarity, the AKT pathway (as well as the TSC1/TSC2 complex) in the regulation of the Wnt/GSK3b/b-Cat pathway, and the BMP pathway in the regulation of PTEN (see text for further discussion). Lastly, both PTEN and mTOR appear to have negative regulatory effects on VEGF through loss of stabilisation of the hypoxia inducible transcription factor 1 (HIF1).

When LKB1 is inactivated we have the following changes observed in a melanocyte. Note the deactivation of normal LKB1 proteins as well as a PTEN loss of function. We then have the models of Bauer and Stratakis, which we graphically depicted before and they are compelling and establish a paradigm which the work of Liu et al can be considered.

Let us go back to LKB1 and its function. From NLM database we have[7]:

LKB1 is a primary upstream kinase of adenine monophosphate-activated protein kinase (AMPK), a necessary element in cell metabolism that is required for maintaining energy homeostasis. It is now clear that LKB1 exerts its growth suppressing effects by activating a group of other ~14 kinases, comprising AMPK and AMPK-related kinases.

Activation of AMPK by LKB1 suppresses growth and proliferation when energy and nutrient levels are scarce. Activation of AMPK-related kinases by LKB1 plays vital roles maintaining cell polarity thereby inhibiting inappropriate expansion of tumour cells. A picture from current

[7] http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=retrieve&dopt=default&rn=1&list_uids=6794

research is emerging that loss of LKB1 leads to disorganization of cell polarity and facilitates tumour growth under energetically unfavorable conditions. Also it is known as PJS; LKB1; hLKB1.

This gene, which encodes a member of the serine/threonine kinase family, regulates cell polarity and functions as a tumor suppressor. Mutations in this gene have been associated with Peutz-Jeghers syndrome, an autosomal dominant disorder characterized by the growth of polyps in the gastrointestinal tract, pigmented macules on the skin and mouth, and other neoplasms. Alternate transcriptional splice variants of this gene have been observed but have not been thoroughly characterized.

From the results of Shaw et al we have[8]:

AMP-activated protein kinase (AMPK) is a highly conserved sensor of cellular energy status found in all eukaryotic cells. AMPK is activated by stimuli that increase the cellular AMP/ATP ratio. Essential to activation of AMPK is its phosphorylation at Thr-172 by an upstream kinase, AMPKK, whose identity in mammalian cells has remained elusive.

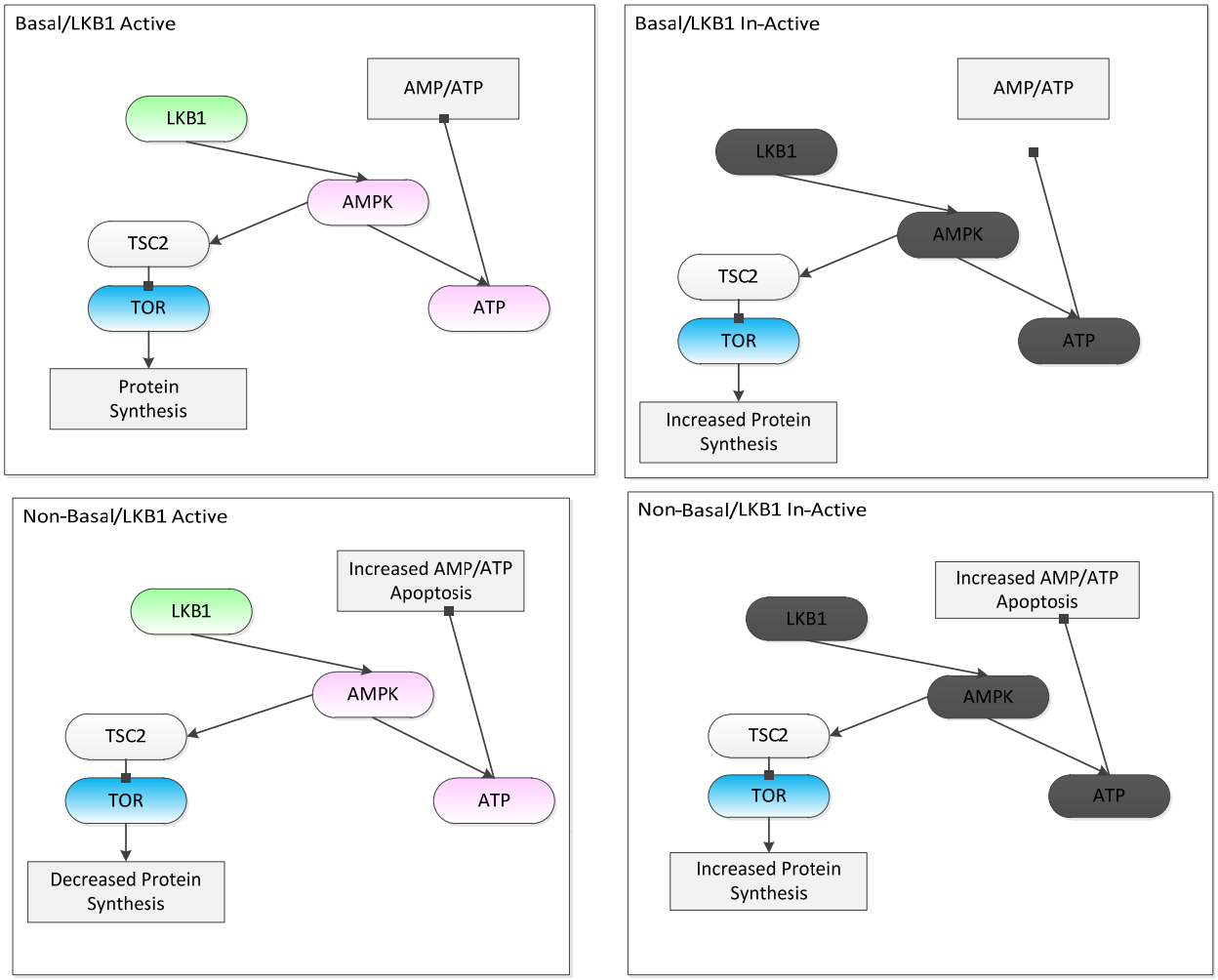
Here we present biochemical and genetic evidence indicating that the LKB1 serine/threonine kinase, the gene inactivated in the Peutz-Jeghers familial cancer syndrome, is the dominant regulator of AMPK activation in several mammalian cell types. We show that LKB1 directly phosphorylates Thr-172 of AMPKalpha in vitro and activates its kinase activity.

LKB1-deficient murine embryonic fibroblasts show nearly complete loss of Thr-172 phosphorylation and downstream AMPK signaling in response to a variety of stimuli that activate AMPK. Reintroduction of WT, but not kinase-dead, LKB1 into these cells restores AMPK activity. Furthermore, we show that LKB1 plays a biologically significant role in this pathway, because LKB1-deficient cells are hypersensitive to apoptosis induced by energy stress.

On the basis of these results, we propose a model to explain the apparent paradox that LKB1 is a tumor suppressor, yet cells lacking LKB1 are resistant to cell transformation by conventional oncogenes and are sensitive to killing in response to agents that elevate AMP. The role of LKB1/AMPK in the survival of a subset of genetically defined tumor cells may provide opportunities for cancer therapeutics.

Also Shaw et al demonstrate several ways in which LKB1 can function when activated in vivo from either a basal or non-basal state. The description can be shown in the following Figure taken from Shaw et (Fig 6 in Shaw et al as modified):

[8] http://www.ncbi.nlm.nih.gov/pubmed/14985505



Shaw et al describe the above as follows:

Model for LKB1 as a sensor of low energy and negative regulator of tumorigenesis and apoptosis. Under basal conditions, LKB1 serves as a sensor of low energy, keeping ATP-consuming processes including protein synthesis in check via AMPK phosphorylation of TSC2.

In response to stresses such as low glucose, hypoxia, nutrient deprivation, or mitochondrial poisons, LKB1 phosphorylates AMPK, which shuts off ATP-consuming processes and up-regulates ATP production to offset the elevated AMP/ATP ratio. This activity prevents the cells from going into apoptosis in response to elevated AMP. In LKB1-deficient cells, under some basal conditions, there may be increases in TOR signaling due to the lack of TSC2 phosphorylation by AMPK, resulting in increased growth or tumorigenic potential. In response to further increases in intracellular AMP, these cells have no mechanism to offset the elevated AMP and go straight into apoptosis.

However, although this is an interesting and compelling description of the metastatic driving factors, there are a multiple set of issues still outstanding:

1. Metastatic behavior implies the ability of the malignant melanocyte to migrate at will within the body. Movement of the melanocyte requires breaking of the E cadherin bonds with the adjacent keratinocytes. Thus is there a sequence of genetic changes and how does this putative mechanism relate to that of the E cadherin mechanism.

As Baas et al state:

A second prominent aspect of polarized simple epithelia is the presence of junctional complexes at the apical boundaries between neighboring cells. These junctions form an impenetrable seal between cells and provide strength to the epithelial sheet by serving as anchoring sites for cytoskeletal elements including the brush border.

We found that LS174T cells do not express junctional proteins, such as ZO-1, and are homozygous mutant for E-cadherin. By contrast, DLD-1 cells are capable of forming tight junctions and adhesion junctions when grown to confluency and appear to express most junctional components already at low-cell density.

We determined the localization of the tight junction component ZO-1 and of the adherens junction protein p120 before and after activation of LKB1 in DLD-1-W5 cells grown at very low density.

2. LKB1 is a gene related to the control from decreased nutrients. However we have the angiogenesis issue related to the increased nutrition of malignant cells. However on the counter side we have the Warburg effect as a counter to normal metabolism, namely cancer cells are anaerobic metabolic systems. What is the balance between the two?

3. Is the LKB1 mutation one of random gene mutations or is it a direct consequence of other downstream mutations? Is perhaps this loss of LKB1 a result of some induced miRNA effect in vivo?

4 THERAPEUTICS

We now want to examine some of the details of each of the two medications and specifically their cellular pathway elements and how putatively the two medications may function. We begin by returning to Danzig et al and seeing what they state about the specifics.

For metformin Danzig et al remark:

Metformin has been shown to inhibit mitochondrial respiration, to induce apoptosis through activation of the AMPK/p53 pathway,

and to trigger a G2-M cell cycle arrest independent of its effect on p53.

Its AMPK activation results in diminished mTOR and S6K1 activity, impeding translation.

Independent of AMPK, metformin also induces G0/G1 cell cycle arrest via reduction of cyclin D1 levels and pRb phosphorylation.

Finally, metformin inhibits nuclear factor κB (NFkB) and Erk 1/2 and reduces levels of c-MYC.

For statins the author's remark:

Statins, through HMG-CoA reductase inhibition, limit mevalonate production, which is used in protein prenylation.

This has been shown to induce apoptosis through Ras inhibition and to reduce invasiveness by preventing intracellular Rho relocalization.

Another cholesterol-dependent effect is statins' interference with lipid raft signaling, which reduces activation of the PI3k/Akt proliferation pathway.

Independent of HMG-CoA reduction, statins can also induce apoptosis through the MEK/ERK pathway, inhibit cell proliferation through blockade of the G1-S and G2-M cell cycle transitions, induce apoptosis by caspase activation and reduce angiogenesis through diminished endothelial nitric oxide production.

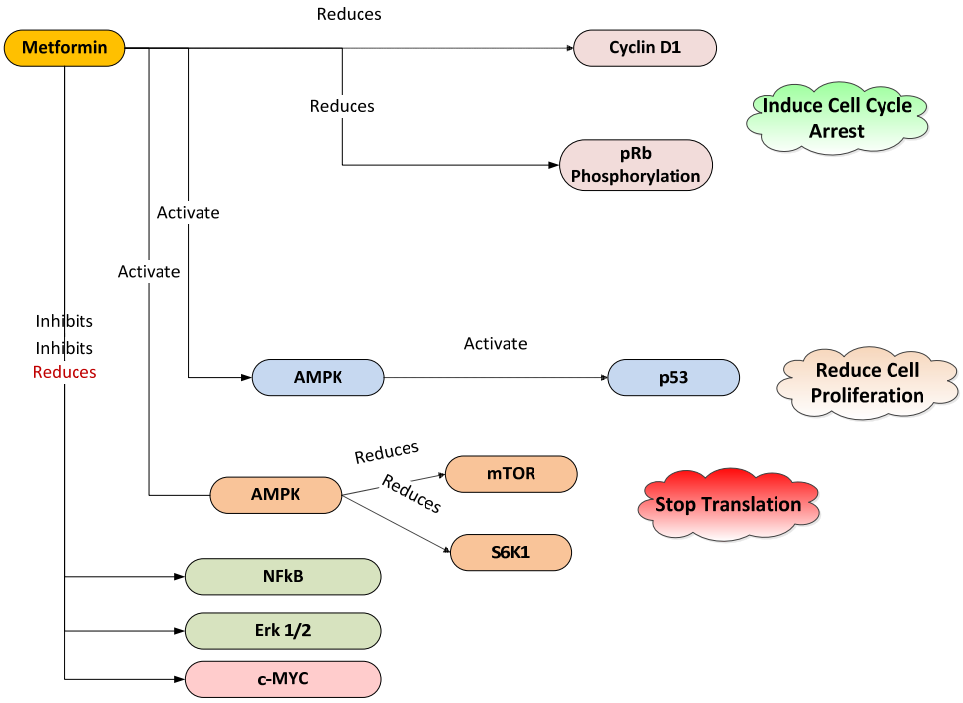
Finally, statins inhibit leukocyte migration and the resultant inflammation, which has been linked to PCa progression.

The statin effects are significant in overall pathway modulation.

4.1 METFORMIN

Metformin is a classic Type 2 Diabetic control medication and has been used extensively with many patients for several decades. We demonstrate below the areas in which Metformin exercises its influence.

It reduces, inhibits, and activates a variety of pathway elements all of which control cell cycles and apoptosis. It controls the metabolic cycles that relate to the pathway elements we have shown in the previous sections.



The impact of AMPK and in turn p53 is a significant pathway. AMPK is as we have seen a significant metabolic player and metformin modulates it behavior. It manages the Cyclin D1 which controls cell cycle growth. One may wonder why so effectively in the prostate, however. The mTOR management is via AMPK as well and then through mTOR C1.

As Mendelsohn et al state:

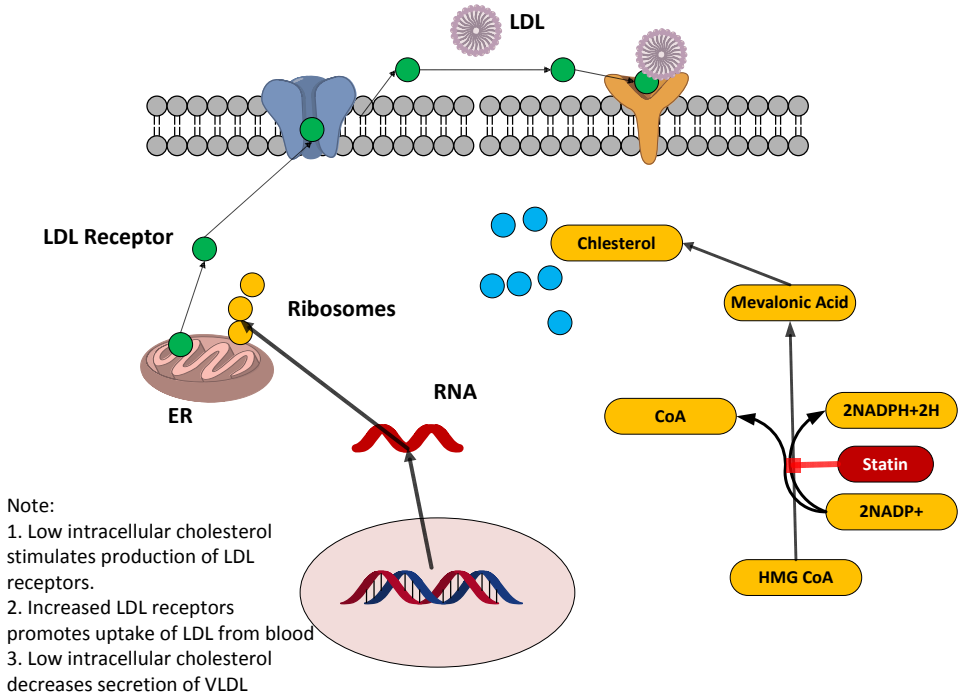
Metformin belongs to the biguanide class of antidiabetic drugs and activates the LKB1/AMPK axis (mediating glucose and energy homeostasis) and inhibits cancer cell viability through the inhibition of mTOR. Metformin can also downregulate mTOR and subsequent cell growth through AMPK-independent mechanisms. A recent study using mouse models of lung cancer to assess the protective effect of metformin suggested two possible mechanisms: decreased levels of circulating insulin and lowered energy stress leading to inhibition of mTOR.

Owing to the fact that studies show metformin is associated with a decreased risk of cancer incidence compared with other treatments (such as insulin) among diabetic patients, metformin is rightfully garnering interest for its role in cancer prevention and therapy and supports further testing in the clinical setting.

The Mendelsohn comment has been demonstrated in Danzig somewhat.

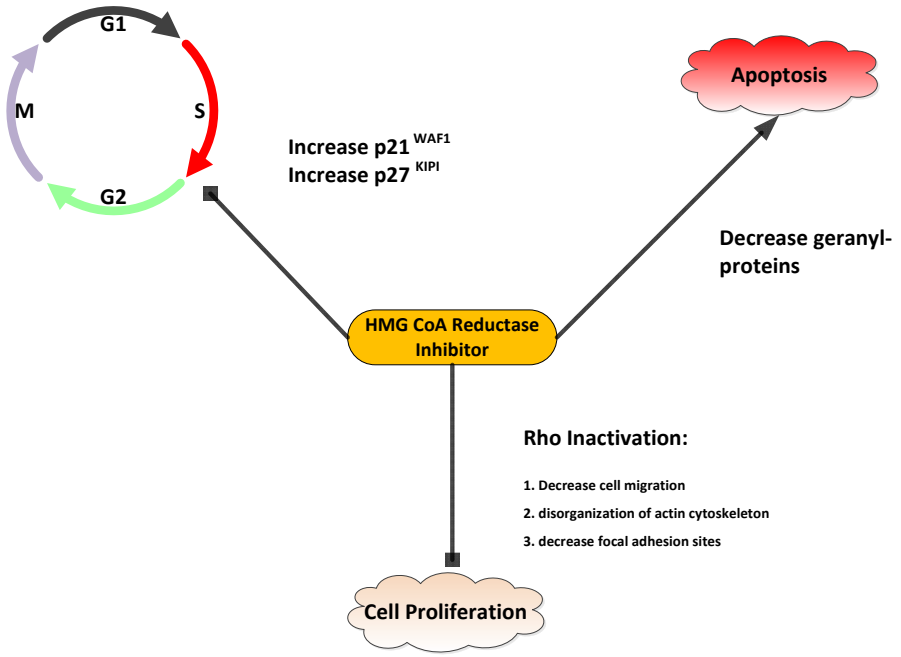
4.2 STATINS

Statins are used to reduce VDL levels. The typical mechanism is shown below. The statin blocks the production of intracellular cholesterol which in turns sets off a cascade that sends out LDL receptors to collect LDL from the blood thus lowering serum LDL.



Overall this is a simple and straightforward mechanism. However, just how this affects the PCa process has been postulated in the paper we have focused on but may be likely to a topic of discussion.

Chan et al have discussed several general mechanisms which are shown below:



As Chan et al note:

HMG-CoA reductase inhibitors have been shown to synchronize tumor cells by blocking the transition of G1-S in the cell cycle, thereby exerting its antiproliferative effect. This effect is reversed with the addition of mevalonate. In primary cultures of human glioblastoma cells, inhibition of Ras farnesylation by lovastatin is associated with reduction of proliferation and migration. However, the inhibition of cell growth by lovastatin may be independent of Ras function .

These findings suggest that geranyl-geranylated proteins (but to a much lesser degree, farnesylated proteins such as Ras) are essential for progression of C6 glioma cells into the S phase of the cell cycle. In addition, N-Ras mutated, primary AML cells were no more sensitive to simvastatin than AML cells without the mutation, suggesting that the inhibition of AML cell proliferation by HMB-CoA reductase inhibitors may be independent of the Ras signaling pathway (54).

On a murine prostate tumor cell line, it was also shown that H-Ras is capable of only inducing cell spreading but incapable of supporting cell proliferation in the absence of geranylgeranylated proteins such as RhoA.

Recently, the antiproliferative effects of HMG-CoA reductase inhibitors on G1-S arrest are thought to be attributable to an increase in p21WAF1/CIP1 and p27KIP1, two cyclin-dependent kinase inhibitors. Rho small GTPase(s), geranyl-geranylated by GGPP, were shown to be important for the degradation of p27KIP1.

The mechanism of HMG-CoA-induced apoptosis also appears to be mediated predominantly through depletion of geranylgeranylated proteins. Add-back experiments of downstream

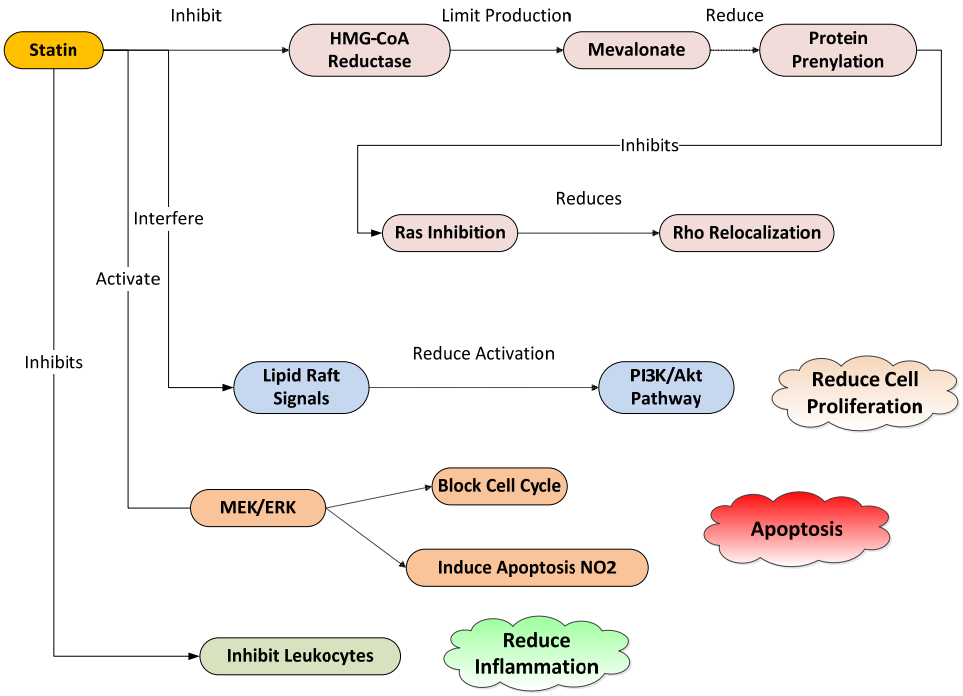
products of the mevalonate pathway were conducted on lovastatin-pretreated human AML cells. Apoptosis induced by lovastatin was abrogated by mevalonate and GGPP and was partially reversed by FPP.

However, other products of the mevalonate pathway, including cholesterol, squalene, lanosterol, desmosterol, dolichol, dolichol phosphate, ubiquinone, and isopentenyladenine, did not affect lovastatin-induced apoptosis in AML cells. Furthermore, the use of a geranylgeranyl transferase inhibitor mimicked the effect of lovastatin on apoptosis, whereas the use of a farnesyl transferase inhibitor was much less effective in triggering apoptosis in AML cells in vitro.

These findings are also supported by a study in colon cancer cells, which showed that addition of GGPP prevented lovastatin induced apoptosis, whereas co-treatment with FPP had no effect. This study also showed that lovastatin treatment resulted in decreased expression of the antiapoptotic protein Bcl-2 and increased the expression of the proapoptotic protein Bax. HMG-CoA reductase inhibitors have also been shown to inhibit cell signaling pathways associated with the invasive and metastatic properties of cancer.

In an in vitro study investigating the effect of HMG-CoA reductase inhibitors on the invasion of human pancreatic cancer PANC-1 cells, fluvastatin markedly attenuated EGF-induced translocation of RhoA from the cytosol to the membrane fraction and actin stress fiber assembly without inhibiting the tyrosine phosphorylation of EGF receptor or cerbB- 2.

From Danzig as modified the control factors associated with statins are shown as follows:



Note that the effects are many and are significant.

5 OBSERVATIONS

The results by Danzig et al present an interesting window to possible control of PCa expansion by using metabolic pathway elements which may also have been causative factors in its initiation. We examine here several observations which may expand the work provided therein.

Let us examine a few additional issues:

What impact will methylation have and is it also driven by similar modalities? We know that methylation is also a factor especially in inflammation like states. Thus what effect does methylation have in this specific case?

Does the process activated by metformin and statins affect all altered prostate cells including stem cells or does it deal solely with the proliferating cells? Here is the issue regarding changes not only to prostate cells but to all cells. There is no specificity of these two therapeutics to prostate cells. The affect cells across the body. Are these effects stabilizing as they may be to the prostate or are they potentially unstabilizing?

How does this combo deal with other cells? This is a corollary to the above observation. Namely here we would examine the impacts, beneficial and harmful, to other cells. These medications are modulating metabolic processes. These metabolic processes are common across many cell areas. It would be useful to see what the balanced effect is.

This must be a common combination. If so that a study may reveal a significant different in end-stage mortality in such a large population. Namely we know that this combination is quite common. If so, then a retrospective study may be beneficial. However, as we have noted before, we do not have either compliance or detailed measurements regarding lipids or blood sugar (eg HbA1c) information.

What is the cause of the synergy between the two? As noted by Danzig et al:

Several potential mechanisms of synergism between the two medications have been explored in preclinical studies. In one study of fatty liver pathogenesis, type 2 diabetic mice fed with a high-fat diet developed increased levels of markers of inflammation and oxidative stress, including C-reactive protein, interleukin-6 and tumor necrosis factor-α. The combinatorial use of atorvastatin and metformin attenuated these effects to a significantly greater degree than either drug alone.

Another study found that the proapoptotic and anti-survival effects of an AMPK activator similar to metformin on malignant melanoma cell lines were enhanced by combination with simvastatin or fluvastatin. As discussed earlier, these two drugs are thought to have a wide range of effects on both metabolic and pleiotropic pathways.

Therefore, the possible means by which they may interact intracellularly to impact cancer behavior are plentiful and diverse.

The cause of the synergy is not really understood. Frankly, even single drug cause is at best generically understood. The range of impact of statins is not fully grasped and thus it may be the statin which has the greater effect. At this stage we need added information regarding the nature of effects.

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