Peer-reviewed version available at *Geriatrics* **2018**, *3*, 73; doi:10.3390/geriatrics3040073

p-21 Activated Kinase as a Molecular Target for Chemoprevention in Diabetes

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Running Title: PAK1 in chemoprevention and diabetes

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ABSTRACT

Hypothesis: Anti-diabetic drugs modulate p-21 activated kinase (PAK) signaling

Introduction: Type 2 diabetes mellitus (T2DM) is a chronic inflammatory disease associated with increased cancer risk. PAK signaling is implicated in cellular homeostasis when regulated, and cancer when unrestrained. Recent reports provided a role for PAK signaling in glucose homeostasis, but the role of PAKs in the pathogenesis of T2DM is unknown. Here we performed a mini-meta analysis to explore if anti-diabetic drugs modify PAK signaling pathways, and provide insight regarding modulation of these pathways to potentially reduce diabetes-associated cancer risk.

Methods: PAK interacting partners in T2DM were identified using online STRING database. Correlation studies were performed via systematic literature review to understand the effect of anti-diabetic drugs on PAK signaling. Mini meta analysis correlated multiple clinical studies and revealed the overall clinical response rate and percentage of adverse events in piogliazone (n=53) and metformin (n=91) treated patients with PAK-associated diseases.

Results: A total of 30 PAK interacting partners were identified (10: reduced beta-cell mass; 10: beta-cell dysfunction; 10: obesity-insulin resistance) which were highly associated with Wnt, and G-protein signaling. Anti-diabetic drug metformin activated signaling pathways upstream; whereas pioglitazone inhibited pathways downstream of PAK. Overall clinical response upon pioglitazone treatment was 53%. 79% of pioglitazone and 75% of metformin treated patients had adverse events. Pioglitazone reduced molecular-PAK biomarkers of proliferation (Ki67 and CyclinD1), and metformin had the opposite effect.

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Conclusions: PAK signaling in T2DM likely involves Wnt and G-protein signaling which may be altered by anti-diabetic drugs metformin and pioglitazone. Apart from the therapeutic limitations of adverse events, pioglitazone may be promising in chemoprevention, however long-term multi-centered studies, which initiate pioglitazone treatment early will be required to fully assess the full potential of these drugs.

Keywords: p-21 activated kinase; pioglitazone, metformin, type 2 diabetes mellitus, cancer, chemoprevention, and inflammation.

INTRODUCTION

Type 2- diabetes mellitus (T2DM) is a global epidemic that significantly reduces the quality of life of geriatric patients, especially in Western society. The etiology of T2DM is intimately linked to obesity, genetics, and sedentary lifestyle. Disease manifestations including blindness and neuropathy are extremely burdensome, and long-term complications such as cardiovascular disease and renal failure, ultimately result in death (McCarthy, 2010).

Although the pathogenesis of T2DM is multifactorial and complex, its current understanding encompasses hepatic insulin resistance, dysfunctional insulin signaling, abnormal glucose metabolism, and persistent hyperglycemia (McCarthy, 2010). In addition to disease specific complications, several consequences of hyperglycemia have been described such as an aberrant immune response, chronic inflammation, and tumorigenesis (Chang & Yang, 2016). Cancer is increasingly common in the elderly, but persistent diabetes also increases the lifelong risk of developing pancreatic, liver, and colorectal cancer and also fuels the tumor microenvironment in cancer patients (Chang & Yang, 2016).

Anti-diabetic drugs such as the biguanides, sulfonylureas, and glitazones have provided the current basis of understanding in the clinical management of T2DM however little is known regarding whether these drugs are also effective in reducing the associated cancer risk. Effective chemoprevention in T2DM will target processes involved in both glucose metabolism and carcinogenesis.

Molecular targets with emerging roles in both cancer and diabetes include a family of six different kinases, the p-21 activated kinases (PAKs) (Dammann et al., 2014). Here we reviewed the literature to further understand PAK signaling

in T2DM. We aimed to correlate the potential effects of anti-diabetic drugs on PAK signaling in order to provide insight on utilizing PAK signaling as a molecular target in cancer chemoprevention in diabetic patients.

METHODS

Molecular analysis of PAK signaling pathways and their involvement in response to anti-diabetic drugs

Systematic literature review and molecular analysis of pre-clinical studies using online library Pubmed (https://www.ncbi.nlm.nih.gov/pubmed) was performed to establish upstream and downstream PAK targets. Key words included (PAK signaling or p21 activated kinases in addition to the following targets: AMPK, RAS, mTOR, PI3K/AKT, RAC1, CDC42, MAPK, p38, JNK, NF-kB, PPAR-gamma, ROS, VEGF, Wnt/Beta-catenin). Similar studies were screened for PAK signaling pathways involving known targets (above) of glucose homeostasis, inflammation, proliferation, survival, and angiogenesis. Both upstream and downstream PAK targets were identified in each of these pathways, and the effect of PAK signaling targets on inflammation, proliferation, survival, and angiogenesis was evaluated. Studies in the literature involving anti-diabetics drugs (glitazones, metformin, glyburide) were further analyzed for their effect on PAK signaling pathways (inflammation, proliferation, survival, angiogenesis) on targets upstream and downstream of PAKs.

Identification of PAK interacting partners

PAK interacting partners were identified using string database (www.string-db.org) as seen in (Szklarczyk et al., 2015)(Campregher et al., 2012). Confidence

was set to 0.40 and active prediction methods, neighborhood, gene fusion, co-occurrence, co-expression, experiments, database, and text mining analysis were performed. Interacting partners were identified in three different conditions which may predispose to T2DM using targets of reduced beta-cell mass (CDKAL1, CDKN2A, CDKN2B), beta cell dysfunction (MTNR1B, TCF7L2, KCNJ11), and obesity/insulin resistance (FTO, IRS1, PPARG) as reported (McCarthy, 2010). Novel PAK interacting partners were further investigated and their pathophysiological role in T2DM was investigated and references for further exploration of targets were provided. All PAK partners were analyzed according to the molecular pathway involved in T2DM.

Clinical study inclusion/exclusion criteria and mini-meta analysis

Human clinical studies were identified on ClinicalTrial.gov. Inclusion criteria consisted of observational or interventional studies using metformin or pioglitazone in patients with diseases previously identified in the literature to have increased PAK expression levels such as bladder, leukoplakia, lung, prostate, esophageal, and colorectal cancer (Dammann et al., 2014). Studies with molecular biomarker analysis or targets downstream of PAKs were included. Studies without data were excluded from analysis. Clinical analyses of overall response rate, adverse events, as well as molecular analysis of biomarkers were performed on pooled data from pioglitazone or metformin treated patients.

RESULTS

PAK signaling is associated with diabetes and cancer

Previous studies have provided a role for PAK in both diabetes and cancer, however a clear overview of the signaling pathways involved in both diseases has not been performed. Systematic review of the literature correlated 14 signaling pathways, which were identified as crucial to PAK, either upstream or downstream of PAK, and 11 or 78% of these pathways were also associated with glucose homeostasis (Table 1 & 2; Supplementary Figure 1 & 2). All of the PAK signaling pathways which were associated with glucose homeostasis were also associated with malignant inflammatory, proliferative, survival, and angiogenic signaling which occurs in diseases such as cancer (Table 3 and Supplementary Figure 3).

PAK interacting partners are associated with the pathogenesis of T2DM

The pathogenesis of T2DM was previously linked to obesity, insulin resistance, and molecular alterations of pancreatic beta cells (McCarthy, 2010). PAK signaling was correlated to pathways involved in glucose homeostasis (Table 2); but whether PAK or its partners are associated in the pathogenesis of T2DM is unknown. We identified protein-protein interactions and prospective interacting partners in the pathogenesis of T2DM by screening PAKs 1-6 and previously identified markers of reduced beta cell mass (Figure 1A), dysfunction (Figure 1B), and obesity and insulin resistance (Figure 1C), using the online STRING database (Szklarczyk et al., 2015).

Thirty total PAK interacting partners were found to involve reduced Beta-cell mass (10), Beta-cell dysfunction (10), and obesity and insulin resistance (10)

(Figure 1D and Supplementary Table 1). Signaling pathways associated with PAK interacting partners included cell cycle control, receptor tyrosine kinases (RTK), G-proteins, and Wnt signaling (Figure 1E). Reduction in Beta-cell mass was exclusively linked to eight-interacting partners involving cell cycle control. Beta-cell dysfunction was found to be associated with four-interacting partners linked to Wnt signaling, and six-interacting partners involving RTKs were associated with obesity/ insulin resistance (Figure 1E). G-protein signaling involved two-interacting partners involved in reduced Beta-cell mass, five-partners in Beta-cell dysfunction, and four-partners in obesity/insulin resistance (Figure 1E). These data suggest PAK interacting partners are associated with and potentially utilize the cell cycle, Wnt, RTK, and G-protein signaling in the pathogenesis of T2DM.

Upstream and downstream PAK signaling pathways are utilized by anti-diabetic drugs pioglitazone and metformin

We found that PAK interacting partners were correlated to the pathogenesis of T2DM, however the role of PAK signaling in T2DM remained elusive. We surveyed this by analyzing the effect of anti-diabetic drugs metformin (met), glyburide (gly), and glitazones (glit) on PAK signaling pathways (Table 4). Overall, glit and met each altered ten PAK signaling pathways whereas gly altered three (Figure 2 A). Further analysis revealed all three anti-diabetic drugs altered pathways upstream and downstream of PAK (Figure 2B). Glit exerted the most profound effect on downstream PAK signaling pathways [glit=9 vs met=7 vs gly=3]. Met altered more pathways upstream of PAK [met=5 vs glit=3 vs gly=1] (Figure 2B).

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Malignant PAK signaling is involved in disease pathogenesis. To further understand the role in which met, gly, and glit interfere with PAK signaling in T2DM, we asked if any of these drugs activate or inhibit inflammatory, proliferative, survival, or angiogenic pathways upstream and downstream of PAK (Figure 2C-F). Differential pathway analysis revealed met and glit consistently altered more pathways than gly (Fig 2C-F). Glit inhibited more pathways downstream of PAK than met in [inflammation: glit=7 vs met=4], [proliferation: glit=8 vs met=4], [survival: glit=8 vs met=4], [angiogenesis: glit=8 vs met=4] (Figure 2C-F). However, met activated more pathways than glit which are exaggerated upstream of PAK and involved in inflammation [met=4 vs glit=2;], proliferation [met=2 vs glit=0], survival [met=3 vs glit=1], and angiogenesis [met=3 vs glit=1] (Figure 2C-F). These data suggest that anti-diabetic drugs might alter PAK signaling. Metformin may activate pathways upstream and glitazones potentially inhibit pathways downstream of PAK.

Pioglitazone and metformin have therapeutic limitations in cancer patients with PAK overexpression

Previous clinical trials have attempted to establish whether met and pio therapeutically have chemopreventive activity in humans, but the results remain highly ambiguous. We showed that met and pio altered PAK signaling upstream and downstream of PAK, and asked whether the chemopreventive nature of these drugs was found in diseases with PAK overexpression. To investigate this, we analyzed clinical studies in diseases known to overexpress PAK in which met or pio treatment was tested (Table 5).

Initially, we screened hundreds of studies on ClinicalTrials.gov for diseases with PAK overexpression and either met or pio treatment. We found one

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observational-prospective cohort and six interventional studies (three single arm and three randomized double blind) with these criteria (Table 5 and Figure 3A).

We investigated the effect of anti-diabetic therapy in patients with PAK over-expression by calculating an overall clinical response rate (OCRR), which was defined as the total number of patients who had ≥ 50% decrease in the sum of all their lesions post-treatment. The OCRR in pio-treated patients was 53% (Figure 3B); 28/53 patients responded and 25/53 did not. The corresponding data for met were unavailable. To further evaluate patient outcome, we calculated the number of serious or other adverse events after pio and met treatment (Figure 3B). Comparison of pio and met treatment revealed a higher percentage of serious [pio: 3/53 = 5.7% vs met: 3/91 = 3.3%] and other [pio: 42/53 = 79% vs met: 68/91 = 74.7%] adverse events upon treatment with pio (Figure 3B). Additionally, in comparison to patients treated with met, a fewer percentage of pio-treated patients were without adverse effects [pio: 11/53 = 21% vs met: 25/91 = 27.4%] (Figure 3B). Pioglitazone associated events included edema (15%), oral pain (13%), and hypertension (7.5%), while those associated with metformin were gastrointestinal symptoms like constipation (7%), diarrhea (23%), and nausea (13%). These data suggest that patients with tumors, which are known to have PAK overexpression, have a good clinical response to pio; and both pio and met treatment, are associated with a high rate of mostly mild adverse events.

Pioglitazone and metformin alter biomarkers downstream of PAK in human disease

We correlated patients which have diseases overexpressing PAK responded to pio, however it was unclear whether pio or met actually interfered with PAK signaling. We therefore analyzed biomarkers downstream of PAK signaling pathway involved in apoptosis, cell cycle, PI3K/mTOR, and PPARγ (Table 6).

We calculated the percent change in biomarker expression in pio and met treated patients to visualize the molecular effect on PAK signaling (Figure 4). Molecular analysis revealed that pio treatment decreased expression levels of multiple biomarkers including, apoptotic marker BCL2, and cell cycle markers CyclinD1 and Ki67, respectively. Pio treatment increased total and cytoplasmic PPARγ levels by 50% and 8%, however it decreased nuclear expression by 32% (Figure 4). Met treatment resulted in an increase in cell cycle biomarker Ki67, and did not alter expression levels of PI3K/mTOR markers PS6K1 or PS65Ser235 (Figure 4). These data indicate that pio inhibited pathways involved in apoptosis and cell cycle regulation whereas met stimulated or did not alter these pathways in patients which potentially have aberrant PAK signaling.

DISCUSSION

Type 2 diabetes is a major cause of disability and death in the elderly worldwide, and in addition to its multiple disease specific complications, it also carries and increased cancer risk, such as colon cancer (Chang & Yang, 2016). P-21 activated kinases are serine-threonine kinases, which influence multiple cell functions from normal cell signaling to cancer (Kumar et al., 2016). Physiological PAK signaling is regulated and implicated in the maintenance of cellular homeostasis, however as the extracellular microenvironment or PAK expression in disease changes, physiological signaling becomes pathological (Figure 5). Here we reviewed physiological PAK signaling and found it is highly correlated with glucose homeostasis, although PAKs role in the pathogenesis of T2DM, and cancer in diabetes patients is largely unknown.

PAK overexpression is associated with disease severity; however, in diabetes, Ahn et al. previously reported a reduction in PAK1 expression in beta cells of the pancreas (Ahn et al., 2016). Here we emphasized the importance of PAK signaling (kinase activity) versus total expression levels, as both chronic inflammation and hyperinsulinemia associated with T2DM (Chang & Yang, 2016) (Donath & Shoelson, 2011), likely leads to pancreatic and peripheral PAK activation. Additionally, six different PAKs have been characterized; therefore signaling from other PAKs may compensate for reduction in PAK1 expression. We identified novel PAK interacting partners associated with the pathogenesis of T2DM, involving reduced beta cell mass and dysfunction, and obesity-insulin resistance (Figure 1). Interestingly, we identified PAK interacting partner IQGAP1 was involved in all three pathways (Figure 1D). IQGAP1 acts as a

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molecular scaffold for small Rho-GTPase activation of PAKs (R. Li et al., 1999), which further implicates the importance of PAK kinase activation in T2DM.

The initial pathogenesis of T2DM involves beta cell expansion to compensate for hyperglycemia, which eventually may lead to reduced beta cell mass (McCarthy, 2010). Here, PAK interacting partners involved with reduced beta cell mass and dysfunction were specifically associated with cell cycle regulation and Wnt signaling (Figure 1D). It is possible that in early diabetes, hyperglycemia promotes cell cycle progression via PAK- Beta-catenin signaling (Park et al., 2012). Over time, PAK driven proliferation and inflammation may lead to oxidative stress (Reuter et al., 2010) and therefore contribute to beta cell dysfunction and subsequent reduction in beta cell mass (Drews et al., 2010). Interestingly, in oxidative stress nuclear Beta-catenin was reported to associate with FOXO transcription factors (Essers et al., 2005), which could potentially modulate PAK expression levels (Mazumdar & Kumar, 2003) in line with reports from Ahn et al. Another explanation for PAK's role here may involve an interaction with tumor suppressor p53 (Figure 1A and D). Interestingly, activation of both p53 and MDM2, a p53 ubiquitin ligase, was reported in T2DM (Xiaomu et al., 2016), and a PAK–MDM2 interaction was previously described (T. Liu et al., 2013). Others provided a role for PAK upstream of p53 (Murray et al., 2010) (Park et al., 2009), thus PAK signaling in T2DM may activate p53 directly, or indirectly via MDM2 or through a cell stress pathway such as oxidative stress or stress associated MAPK like p38/JNK (Shi et al., 2014) thereby inducing apoptosis, impeding cell cycle progression, and subsequently reducing beta cell mass. Additional PAK interacting partners associated with pathways involving obesity-insulin resistance involved targets in RTK signaling, which is likely a

consequence of multiple growth factors, and the chronic inflammatory state associated with obesity in T2DM (Donath & Shoelson, 2011).

We sought to illuminate the role of PAK signaling in T2DM by analyzing the effect that three well-known classes of anti-diabetic drugs had on PAK signaling (Figure 2). Biguanide (metformin), sulfonylurea (glyburide), and glitazone (pioglitazone) all interfered with signaling upstream and downstream of PAKs, and this effect was more apparent with metformin (upstream) and pioglitazone (downstream) of PAKs (Figure 2). Both metformin and pioglitazone mediated inflammatory, proliferative, survival, and angiogenic pathways associated with PAKs (Table 4 and Figure 2). However it is important to note that even though PAK signaling appears to be modulated by these drugs, one major limitation of our study is a lack of an experimental model to support this, therefore we can only speculate using the evidence we found in the literature.

Considering the role of PAK signaling in the initiation of disease (Kumar et al.,, 2016), and that activation of PAK signaling was correlated to T2DM (Table 2), and potentially inhibited anti-diabetic drugs (Figure 2), we asked whether metformin or pioglitazone were beneficial in patients with PAK-overexpressing diseases including oral cancer (Parvathy et al., 2016), non small cell lung cancer (Y. Liu et al., 2016), prostate (T. Liu et al., 2013), esophageal cancer (Gan et al., 2015), bladder (Ito et al., 2007), and colorectal cancer (Song, Wang, Zheng, Yang, & Xu, 2015). Pre-clinical studies investigating the chemopreventive effects of these drugs seemed promising (Girnun et al., 2002) (Grommes et al., 2013) (Burotto & Szabo, 2014), however results from human studies remain highly ambiguous (Lewis et al., 2015) and a more complete analysis of this data would allocate whether inhibition of PAK signaling pathways by metformin or

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pioglitazone is a promising for chemoprevention. We utilized a mini-meta-analysis of several studies (Table 5) involving metformin and pioglitazone use in cancer patients to investigate whether these treatments are potential candidates to target PAK signaling (Figure 3). However, it is important to note our findings here are only speculation and we cannot definitively conclude PAK is truly altered by met or pio in these reviewed studies. Also, modulation of PAK signaling by these agents is largely dependent on a particular cell type and tissue context, which is lost when combining studies with different diseases. Nonetheless, our analysis was still able to link that pioglitazone treated patients had a clinical response rate of 53% in PAK dependent diseases (Figure 3B). It was unfortunate that data needed to calculate response rate to metformin were not available for analysis. Both pioglitazone and metformin treatment resulted is relatively few serious adverse events. However, the overwhelming majority of patients, nearly 80% of pioglitazone and 75% metformin treatment patients had mild adverse events (Figure 3B). The high number of pioglitazone associated events such as edema, and hypertension were likely due to the advanced treatment regimen used in these patients, equivalent to 45mg/day, versus the standard care at a dose equivalent to 15mg/day (Majima et al., 2006). Other studies have indicated fewer adverse events at lower doses equivalent to 7.5mg/day (Majima et al., 2006). Although gastrointestinal symptoms like constipation, diarrhea, and nausea are common side effect of metformin (Bosi, 2009), so many adverse events were unexpected and likely not attributable to dose, as patients received a standard of care equivalent of 2000mg/day. Given that metformin is the gold standard in treatment of T2DM (Bosi, 2009), the frequency of adverse events is concerning. Although not life threatening,

adverse events are a serious therapeutic limitation and concern for future chemopreventive studies, as long-term patient compliance will dramatically decline if quality of life is decreased by therapy. In addition to our analysis, ten-year treatment of pioglitazone in diabetic patients was associated with increased prostate and pancreatic cancer risk, (Lewis et al., 2015), making its long-term use in chemoprevention questionable.

We asked whether PAK signaling was even affected in these patients by pioglitazone or metformin at the molecular level (Table 6 and Figure 4), and found that pioglitazone but not metformin decreased PAK signaling. This data was in line with our analysis of signaling pathways affected by these drugs (Table 4), in that metformin induced signaling upstream of PAKs while pioglitazone rather reduced downstream signaling (Figure 2). Here pioglitazone reduced markers of proliferation including cyclinD1 and Ki67, while metformin had the opposite effect on proliferation. Long-term treatment with metformin in diabetic patients may therefore induce, not inhibit long-term cancer risk, however future studies would need to investigate this further. However, recent work of Bradley and others show metformin may have long-term chemopreventive effects in preventing colorectal cancer in male diabetic patients (Bradley et al., 2018). Due to our small sample size in this study we were unable to divide or based on gender or colon cancer, which may have masked similar findings.

Considering our clinical and molecular analysis of PAK biomarkers in pioglitazone treated patients, future studies should investigate the long term side effects associated with its treatment and whether PAK signaling in disease can be impeded early on, to block malignant transformation (Kumar et al., 2016).

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Although PAK signaling appears to promote malignant disease, whether inhibition of PAK will exacerbate diabetes in these patients is of concern. Each case will ultimately need to be analyzed on a risk-reward basis, and patients with both diabetes and increased cancer risk such as (BRCA/Lynch Syndrome/FAP) would be more favorable candidates for long-term studies with these potentially chemopreventive agents.

This was the first study to provide a mechanistic explanation of how anti-diabetics may target PAK signaling for their potential use for chemoprevention in patients with T2DM, albeit our signaling pathway analysis included multiple pre-clinical studies, which were not of high significance. Ideally, a thorough signaling pathway analysis should use PAK1 and p-PAK1 as biomarkers in multiple human studies, however this data was unavailable for analysis due to the limitations of the current literature in regard to our highly specific question (Dammann et al., 2015). Considering the few patients analyzed here, the accuracy of this data is supported by its correlation between literature reports (Table 4 and Figure 2) and our analysis of PAK signaling pathways from human studies (Table 6 and Figure 4). Future studies with more patients and additional readouts of known PAK targets would provide a more clear analysis of whether chemoprevention in diabetes with pioglitazone is feasible long term.

Future directions

Although, not associated with diabetes, other studies of chemoprevention have shown that anti-inflammatory drugs like (aspirin in colorectal cancer), and (mesalamine in colitis associated cancer), reduce cancer-associated risk (Drew et al., 2016) (Lyakhovich & Gasche, 2010). Mesalamine, the first line treatment for

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chronic inflammation in ulcerative colitis (Karagozian & Burakoff, 2007), was recently established as a PAK1 inhibitor (Khare et al., 2013), and others have shown it is a PPAR-gamma ligand (Rousseaux et al., 2005); and both of these mechanisms are in line with glitazones (Dammann et al., 2015). Therefore, future directions in chemoprevention in diabetes should analyze PAK expression/ phosphorylation upon glitazone treatment and see if the effects are similar to those of mesalamine in impeding chronic inflammation in colitis associated colon cancer.

Concluding remarks

Anti-diabetics like pioglitazone or metformin should be utilized as a platform for further understanding the role of PAKs as a chemopreventive target in diabetes; however, before this is possible, future studies must standardize doses specific to the associated disease in order to modulate PAK signaling appropriately and minimize adverse effects. Ideal chemoprevention, like mesalamine, will block inflammation, and impede aberrant PAK signaling without altering cellular homeostasis.

Disclosure: This manuscript was compiled as a research and literature review at the Medical University of the Americas (MUA). MUA accepted the terms and conditions required to further utilize this work for publication purposes.

Funding: This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

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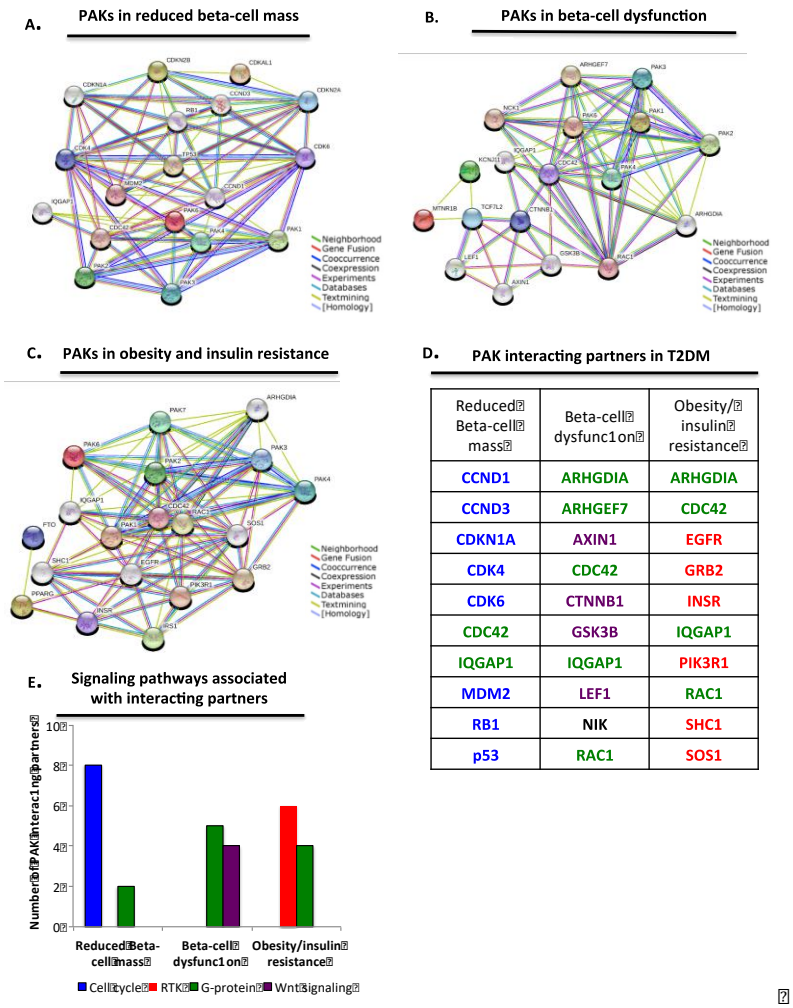

Reduced Beta-cell mass	Beta-cell dysfunction	Obesity/ insulin resistance
CCND1	ARHGDIA	ARHGDIA
CCND3	ARHGEF7	CDC42
CDKN1A	AXIN1	EGFR
CDK4	CDC42	GRB2
CDK6	CTNNB1	INSR
CDC42	GSK3B	IQGAP1
IQGAP1	IQGAP1	PIK3R1
MDM2	LEF1	RAC1
RB1	NIK	SHC1
p53	RAC1	SOS1

Figure 1 (A-E)

PAK interacting partners are associated with pathogenesis of T2DM. PAK interacting partners were identified with sting-db.org using known targets involved in **A)** reduced beta-cell mass, **B)** beta-cell dysfunction, and **C)** obesity/insulin resistance. **D)** Novel PAK interacting partners involved in reduced beta-cell mass, beta cell dysfunction, and obesity/ insulin resistance are displayed and color coded based on their involvement in the cell cycle (blue); G-proteins (green); receptor tyrosine kinase (red); and Wnt signaling (purple). **E)** Bar graphs indicate the number of interacting partners involved in pathway associated with pathogenesis of T2DM. Reduced beta cell mass is associated with the cell cycle, beta cell dysfunction is associated with Wnt signaling, and obesity/

insulin resistance is associated with receptor tyrosine kinases. G-proteins are associated with all three pathways leading to T2DM.

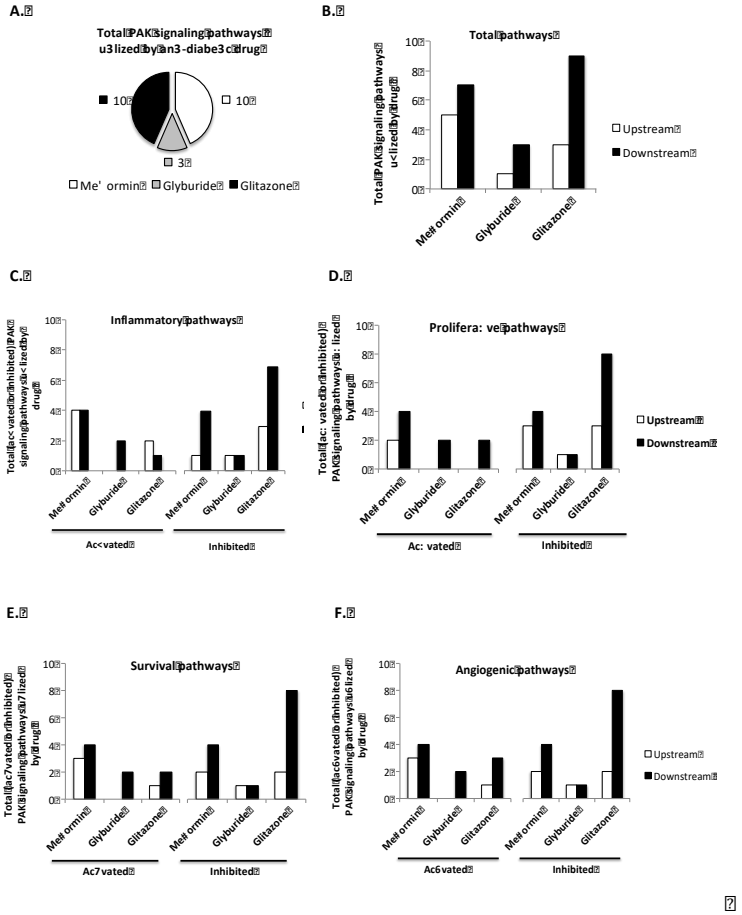

Figure 2 (A-F)

Anti-diabetic drugs utilize upstream and downstream PAK signaling pathways. A) Pie graph indicates total number of PAK signaling pathways utilized by metformin (met), glyburide (gly), or glitazone (glit). **B)** Total number of pathways utilized by anti-diabetic drugs upstream or downstream of PAK. Note that total number of pathways in A) and B) are not equal as pathways interfered by drug may involve targets both up and downstream PAK. All bar graphs indicate the number of PAK signaling pathways involved in inflammation **(C)**, proliferation **(D)**, survival **(E)**, and angiogenesis **(F)** upstream or

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downstream of PAK, which are either activated or inhibited by anti-diabetic drugs.

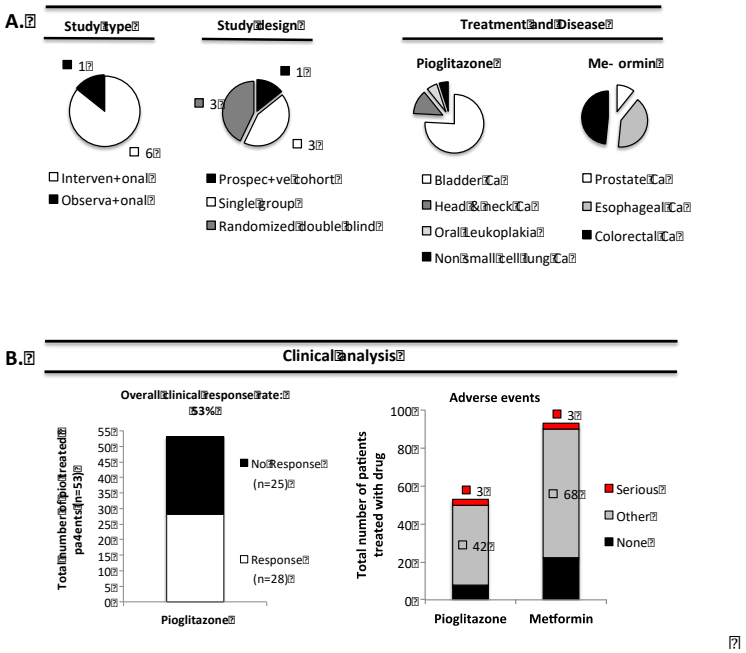


Figure 3 (A-B)

Pioglitazone and metformin have therapeutic limitation in cancer patients. **A)** Pie graphs demonstrate study type, design, and disease associated with their respective treatment. **B)** Clinical analyses included calculation of overall response rate and number of serious or other adverse events in pio and met treated patients. Results are pooled data from 53 pio treated patients [NCT00099021 (n=21), NCT00951379 (n=26), NCT01342770 (n=6)] and 91 met treated patients [NCT01433913 (n=10), NCT01447927 (n=36), NCT01312467 (n=45)]. Serious or other adverse events were defined based on ClinicalTrial.gov.

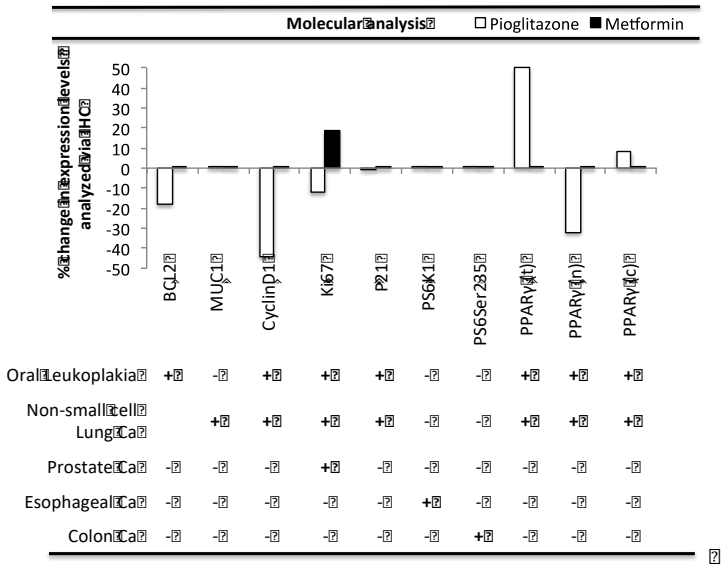


Figure 4

Pioglitazone and metformin alter biomarkers downstream of PAK in human disease. Bar graphs are data representing expression of biomarkers calculated by IHC in patients treated with (+ or -) pio [NCT00951379 (n=25), NCT01342770 (n=5)] or met [NCT01433913 (n=8), NCT01447927 (n=36), NCT01312467 (n=32)].

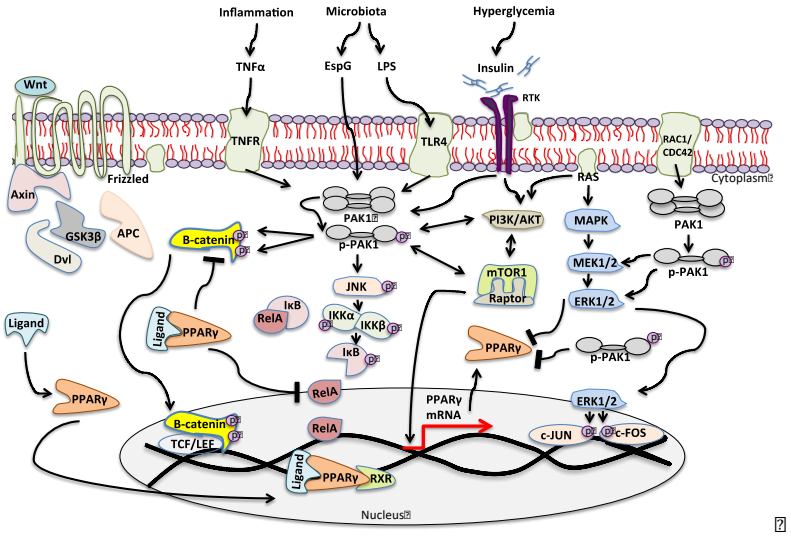
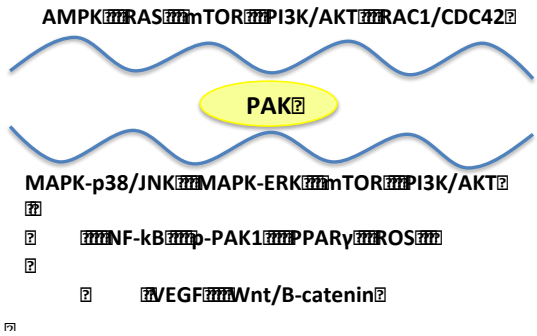


Figure 5

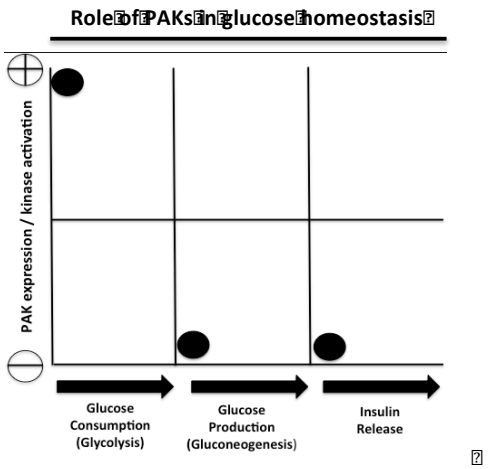
Role of PAK signaling in response to inflammation, altered microbiota and hyperglycemia. Image is read from left to right. (**Far left)** Binding of a Wnt ligand to the Frizzled receptor disrupts the multi-subunit destruction complex consisting of Axin, Dvl, GSK3beta, and APC, which normally tags Beta-catenin to the proteasome. Here, Beta-catenin is no longer degraded but moves to the nucleus where it binds to TCF/LEF and initiates transcription of target genes involved in cell cycle progression, proliferation and survival. **(Low left)** Ligand binding to PPAR-gamma results in its activation and nuclear translocation where it then binds RXR and induces its own transcription as well as genes involved in glucose homeostasis and lipid transport. PPARy directly inhibits Beta-catenin and the NF-kB subunit RelA. **(Central)** Inflammatory cytokines like TNFalpha or microbial products such as EspG or lipopolysaccharides (LPS), result in PAK1 activation. PAK1 phosphorylates Beta-catenin and leads to its stabilization and full transcriptional activation. PAK1 phosphorylates JNK and activates the IKK complex which disrupts RelA from IkB and leading to transcription of genes involved in inflammation and survival. Hyperglycemia results in insulin

stimulation of RTKs which further activate PAK1 or directly contribute to a PI3K/AKT/mTOR pathway. PAK1 may also directly contribute to activation of PI3K or mTOR or vice versa. Note that activation of the mTOR1-Raptor complex leads to transcription of PPAR-gamma. As PPAR-gamma levels increase they fulfill their role in the nucleus and cytoplasm. RAS is a key component of the MAPK and PI3K pathway. MAPK-ERK signaling results in activation of JUN and FOS (nucleus) and inhibits PPAR-gamma (cytoplasm). **(Far right)** RAC1/CDC42 are small Rho-GTPases, which also lead to PAK1 activation. PAK1 stimulates the MAPK-ERK cascade and also inhibits PPAR-gamma.



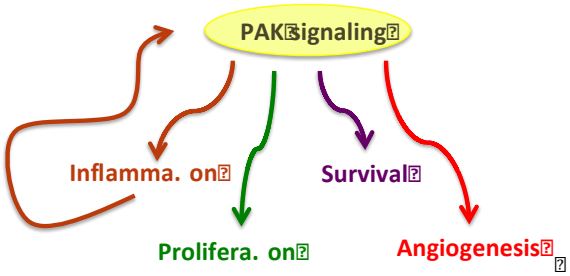
Supplementary Figure 1

Role of signaling pathways upstream and downstream of p-21 activated kinases. (Top) Pathways above PAK in figure are associated with PAK activation. (Bottom) PAK activation contributes to the activation of these pathways. Multiple signals lead to PAK activation via the small Rho G-proteins RAC1/CDC42, or RTK activation of membrane bound RAS. Although mTOR and PI3K/AKT pathways both converge upon PAK activation, PAK activation may also contribute to their activation. Other reports have described that AMPK signaling leads to PAK activation (Dammann et al., 2014). Depending on the cell type and environmental signal, PAK phosphorylates or scaffolds its targets and contributes to MAPK signaling, including both ERK and p38/JNK, PI3K/AKT/mTOR, NF-kB, PPAR-gamma, ROS, Wnt-Beta catenin, and VEGF (Dammann et al., 2014).



Supplementary Figure 2

Diverse roles of PAK in glucose homeostasis. Changes in PAK expression or kinase activity alter glycolysis, gluconeogenesis, and insulin release. PAK overexpression impedes glycolysis and PAK inhibition impairs gluconeogenesis and insulin release (Shalom-Barak & Knaus, 2002)(Z. Wang, Oh, & Thurmond, 2007)(Z. Wang & Thurmond, 2010).



Supplementary Figure 3

PAK signaling in disease. PAK overexpression leads to aberrant PAK signaling and drives cellular inflammation, proliferation, survival, and angiogenesis. Chronic inflammation also drives PAK signaling pathways.

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Upstream	
AMPK	(Kong et al., 2016)
RAS	(Menard & Mattingly, 2003)
mTOR	(Gu et al., 2013)(Ishida, Li, Yi, & Lemon, 2007)
PI3k/ AKT	(Ijuin & Takenawa, 2012)
RAC1	(Shin, Kim, Roy, & Kim, 2013)
CDC42	(Shin et al., 2013)

Downstream	
MAPK-p38/JNK	(Dodeller & Schulze-Koops, 2006)
MAPK-ERK	(Huynh, Liu, Baldwin, & He, 2010)
mTOR	(Khare et al., 2015)
PI3k/ AKT	(Huynh et al., 2010) (Khare et al., 2015)
NF-kB	(Dammann, Khare, Lang, et al., 2015)
p-PAK1	(Dammann, Khare, Lang, et al., 2015)
PPARy	(Dammann, Khare, Lang, et al.,
ROS	(DeSantiago et al., 2014)
VEGF	(Huynh et al., 2010)
Wnt/B-catenin	(Khare et al., 2013) (Zhu et al., 2012)(M.-H. Park et al., 2012) Zhen et al.

Table 1

Upstream and downstream PAK signaling pathways. Upstream (Left) pathways result in PAK activation. Activated PAK contributes to multiple downstream signaling pathways (Right).

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PAK signaling pathways in glucose homeostasis	
	Reference
AMPK	(Kong et al., 2016)(Viollet et al., 2009)
MAPK-p38/JNK	(Sozen, Ozturk, Yaba, & Demir, 2015)
MAPK-ERK	(Zhang, Thompson, Hietakangas, & Cohen, 2011)
mTOR	(Altomare & Khaled, 2012)
NF-kB	(Mauro et al., 2011)
p-PAK1	(Yu-ting Alex Chiang & Jin, 2014)
PI3K/AKT	(Sylow et al., 2014)
PPARy	(Picard & Auwerx, 2002)
RAC1/CDC42	(Gautam, Ishrat, Singh, Narender, & Srivastava, 2015)(Ahn et al., 2016)
RAS	(Manchester, Kong, Lowry, & Lawrence, 1994)
Wnt/Beta-catenin	(Elghazi et al., 2012)

Table 2

PAK signaling pathways are involved in glucose homeostasis. The role of PAK in glucose homeostasis is complex. Each PAK signaling pathway plays a unique part in glucose homeostasis, and significant overlap exists between each of the pathways listed.

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Role of PAK signaling pathways in disease					
Pathway	Inflammation	Proliferation	Survival	Angiogenesis	Reference
AMPK	↓	↓	↑	↑	(Salminen, Hyttinen, & Kaarniranta, 2011) (Motoshima, Goldstein, Igata, & Araki, 2006) (Domenech et al., 2015) (Ouchi, Shibata, & Walsh, 2005)
MAPK-p38/JNK	↑	↑	↑	↑	(Cuadrado & Nebreda, 2010)
MAPK-ERK	↑	↑	↑	↑	(Huynh et al., 2010)
mTOR	↑	↑	↑	↑	(Karar & Maity, 2011)
NF-kB	↑	↑	↑	↑	(Dammann, Khare, Lang, et al., 2015)
p-PAK1	↑	↑	↑	↑	(Dammann, Khare, Harpain, et al., 2015)
PI3k/AKT	↑	↑	↑	↑	(Karar & Maity, 2011)
PPARy	↓	↓	↓	↑↓	(Salomone, 2011); (Kotlinowski & Jozkowicz, 2016)
RAC1/CDC42	↑	↑	↑	↑	(Ma et al., 2013)
ROS	↑	↑↓	↑↓	↑↓	(Liou & Storz, 2010); (DeSantiago et al., 2014)
VEGF	↑	↑	↑	↑	(DEVERY et al., 2015);(Ma et al., 2013); (Xuri Li et al., 2009)
Wnt/B-catenin	↑	↑	↑	↑	(Dammann, Khare, Harpain, et al., 2015), (Khare et al., 2015) (Zhu et al., 2012), (M.-H. Park et al., 2012)

Table 3

Role of PAK signaling in disease. PAK activation or overexpression in malignant disease results in activation of multiple signaling pathways, which drive inflammation, proliferation, survival, and angiogenesis. Arrows indicate the effect of signaling pathways on inflammation, proliferation, survival and angiogenesis.

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PAK signaling pathways utilized by anti-diabetic drugs				
Pathway	Metformin	Glyburide	Glitazone	Citation
AMPK	↑	-	↑	(You et al., 2013) (Coletta et al., 2009)
MAPK-P38/JNK	↑	↑	↓	(Wu et al., 2011) (A. Kumar, Al-Sammarraie, DiPette, & Singh, 2014);(Qian et al., 2008) (Okami et al., 2013)
MAPK-ERK	↓	-	↑↓	(A. Kumar et al., 2014);(Bolden, Bernard, Jones, Akinyeke, & Stewart, 2012); (Wei, Ma, Xu, Zhang, & Tang, 2016)
mTOR	↓	-	↓	(Nair et al., 2014); (San, Liu, Zhang, Shi, & Zhu, 2015); (Blanchard et al., 2012)
NF-kB	↓	-	↓	(Isoda et al., 2006);(Hou, Moreau, & Chadee, 2012); (Dammann, Khare, Lang, et al., 2015)
p-PAK1	↑	-	-	(You et al., 2013)
PI3K/AKT	↑	↓	↓	(Xu et al., 2016); (Qian et al., 2008)
PPAR-y	-	-	↑	(Picard & Auwerx, 2002)
RAC1/CDC42	↑	-	-	(A. Kumar et al., 2014), (A. Kumar et al., 2014)
RAS	↓	-	-	(Nair et al., 2014)

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ROS	-	↑	↓	(Qian et al., 2008); (Yuan et al., 2011)
VEGF	↑↓	-	↑↓	(Dallaglio et al., 2014);(Ersoy et al., 2008); (Yuan et al., 2011), (Kotlinowski & Jozkowicz, 2016)
Wnt/B-catenin	-	-	↓	(D. Lu & Carson, 2010)(D. Lu & Carson, 2010), (P.-S. Wang et al., 2009).

Table 4

PAK-signaling pathways are utilized by anti-diabetic drugs. The effect of anti-diabetic drugs metformin, glyburide, and pioglitazone on PAK signaling pathways as reported by the literature. Arrows indicate the effect of drug on PAK signaling pathway ↑ (increases); ↓ (decreases); ↑↓ (both).

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Clinical studies using pioglitazone or metformin in diseases with PAK overexpression						
Study type	Design	Disease	Drug	Primary outcome measure	Patients: treatment (tx); control (con)	Study ID
Observational	Prospective cohort	Diabetes Bladder Ca	pio	Incident Diagnoses	Tx: 34181 Con: 158918	NCT01637935
Interventional	Single group; prevention	Head/Neck Ca; Oral Leukoplakia	pio	Overall response	Tx: 21 Con: 0	NCT00099021
Interventional	Randomized; double blind; treatment	Oral Leukoplakia	pio	Overall response	Tx: 27 Con: 25	NCT00951379
Interventional	Single group; Treatment	Non Small cell lung Ca	pio	% change Ki67 IHC	Tx: 6 Con: 0	NCT01342770
Interventional	Randomized; double blind; treatment	Prostate Ca	met	% change Ki67 IHC	Tx: 10 Con: 10	NCT01433913
Interventional	Randomized; double blind; prevention	Barrett Esophagus; Esophageal Ca	met	% change pS6K1 IHC	Tx: 38 Con: 36	NCT01447927
Interventional	Single group; prevention	Adenomatous polyp; CRC; obesity	met	% change S6- serine-235 IHC	Tx: 45 Con: 0	NCT01312467

Table 5

Clinical studies using pioglitazone or metformin in diseases with PAK overexpression. Overview of clinical studies analyzed for their effect of anti-diabetic drug pioglitazone (pio) or metformin (met) on PAK signaling pathways in diseases known to overexpress PAK. All clinical data is accessible at ClinicalTrials.gov using the indicated study ID.

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Biomarker analysis in cancer patients treated with pioglitazone or metformin													
Disease	Drug		Marker analyzed via immunohistochemistry (IHC)										Study ID
			Apoptosis		Cell cycle control			PI3K/ mTOR		PPARy			
	pioglitazone	metformin	BCL2	MUC1	CyclinD1	Ki67	P21	PS6K1	PS6ser235	Total	Nuclear	Cytoplasm	
Oral leukoplakia	+	-	↓↓	-	↓↓	↓	↑	-	-	-	↓↓↓	↑	NCT00951379
Non-small cell lung Ca	+	-	-	0	↓↓↓↓	↓↓	↓	-	-	↑↑↑↑	-	-	NCT01342770
Prostate Ca	-	+	-	-	-	↑↑	-	-	-	-	-	-	NCT01433913
Esophageal Ca	-	+	-	-	-	-	-	↑	-	-	-	-	NCT01447927
Colon Ca	-	+	-	-	-	-	-	-	0	-	-	-	NCT01312467

Table 6

Pioglitazone and metformin alter biomarkers downstream of PAK in human disease. Clinical trials, which utilized pio or met in diseases with PAK overexpression, were analyzed for their IHC data. Biomarkers: apoptosis (BCL2, MUC1), cell cycle control (CyclinD1, Ki67, P21), PI3K/mTOR (PS6K1, PS6Ser235), and PPARy. Symbols indicate (+) treatment or (-) not available. (↑; ↓; 0) indicates percent increase or decrease in expression analyzed via IHC. 0 (no change); 1 arrow (> 1% change): 2 arrows (>10% change); 3 arrows (>20% change); 4 arrows (>50% change).

Pathophysiological role of interacting partners in T2DM		
Target	**Description**	**Reference**
ARHGDIA	Rho GDP-dissociation inhibitor 1: RAC1/CDC42 inactivation	(W. Lu et al., 2016)
ARHGEF7	Rho guanine nucleotide exchange factor 7: RAC1 activation	(Nola et al., 2008)
AXIN1	Component of Beta-catenin destruction complex in Wnt signaling	(Clevers, Loh, & Nusse, 2014)
CCND1	G1/S specific cyclin-D1: RB inactivation and promotion of G1/S transition	(Taneera et al., 2013)
CCND3	G1/S specific cyclin-D3: RB inactivation and promotion of G1/S transition	(Marselli et al., 2010)
CDKN1A	Cyclin dependent kinase inhibitor 1: impedes cell cycle progression	(Taneera et al., 2013)
CDK4	G1/S specific cyclin dependent kinase 4: RB inactivation and promotion of G1/S transition	(Taneera et al., 2013)
CDK6	G1/S specific cyclin dependent kinase 6: RB inactivation and promotion of G1/S transition	(Taneera et al., 2013)
CDC42	Cell division control protein 42: GTPase which activates PAKs	(Raut et al., 2015)
CTNNB1	Component of Wnt signaling and adherent junctions at cell membrane	(Clevers et al., 2014)

EGFR	Epidermal growth factor receptor: receptor tyrosine kinase activates RAS, MAPK, PAK1, PI3 kinase	(Tomar & Schlaepfer, 2010)
GRB2	Growth factor receptor bound protein 2: adaptor protein linking EGFR to RAS	(Puto, Pestonjamasp, King, & Bokoch, 2003)
GSK3B	Component of Beta-catenin destruction complex	(Clevers et al., 2014)
INSR	Insulin receptor: receptor tyrosine kinase activates MAPK and PI3K	(Yuting Alex Chiang, Shao, Xu, Chernoff, & Jin, 2013)
IQGAP1	RAS GTPase activating like protein: scaffolds CDC42 for cytoskeletal reorganization	(R. Li et al., 1999)
LEF1	Lymphoid enhancing binding factor 1: transcription factor downstream of Wnt/Beta-catenin signaling	(Clevers et al., 2014)

MDM2	E3 ubiquitin-protein ligase: induces proteasomal degradation of p53 , RB, IGF1R	(Girnita, Girnita, & Larsson, 2003)
NIK	NF-kB inducing kinase: mediates activation of NF-kB in inflammation	(Neumann, Foryst-Ludwig, Klar, Schweitzer, & Naumann, 2006)
PIK3R1	Phosphatidylinositol 3-kinase regulatory subunit alpha: mediates glucose uptake in adipose and skeletal muscle	(Y. T. A. Chiang et al., 2014)
p53	Cellular tumor antigen p53: induce cell cycle arrest	(Kung & Murphy, 2016)
RAC1	Ras-related C3 botulinum toxin substrate 1: GTPase which activates PAK1	(Sylow et al., 2014)
RB1	Retinoblastoma-associated protein: tumor suppressor which impedes G1/S phase transition	(Moreno-Navarrete et al., 2013)
SHC1	SHC-transforming protein 1: adaptor which coordinates growth factor signaling pathways	(Wagner et al., 2004)
SOS1	Son of sevenless homolog 1: activation of RAS	(Barroso et al., 2003)

Peer-reviewed version available at Geriatrics 2018, 3, 73; doi:10.3390/geriatrics3040073

Supplementary Table 1

The pathophysiology of PAK interacting partners in T2DM. Targets identified using string-db.org were further investigated and a short description of their pathophysiology is shown.