Journal of Research and Practice on the Musculoskeletal System
JRPMS

Review Article

Signaling pathways that overactivate metabolism and drive neoplasia, in rhabdomyosarcoma

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Abstract

The functional status of a cell is expressed in its metabolic activity. Cancer cells differ from normal cells through unlimited cell division, and show a greater need for energy for their rapid growth and duplication. Thus, cancer-associated metabolic alterations, i.e. metabolic over-activation through signaling pathways alterations, have emerged as a cancer hallmark. Rhabdomyosarcoma (RMS) is a myogenic tumor classified as the most frequent soft tissue sarcoma affecting children, adolescents and adults. Signaling pathway alterations present in this cancer can be related to increased metabolic activity and drive neoplasia. In this review first of all, we would like to enlighten cancer and particular RMS metabolism. Further, we aim to summarize several pathways related to oncogenic drivers affecting metabolism of RMS cells in order to simulate them (in other studies) with a system biology approach. The understanding of the common mechanisms that transform physiological cells to malignant may reveal novel therapeutic targets and strategies that may improve the currently poor outcome for patients with RMS.

Keywords: Signaling, Pathway, Metabolism, Neoplasia, Rhabdomyosarcoma

Introduction

In several research studies, evidence emerged that at the molecular level most chronic diseases, including cancer, are caused by a deregulated inflammatory response. Characterization of protein families of transcription factors NF-κB, AP-1 and STAT3 which have gene targets that include inflammatory mediators and adhesion molecules provided the molecular basis for the role of inflammation in cancer: many of those inflammatory mediators influence transendothelial migration of inflammatory cells and vascular permeability. Additionally, molecules normally expressed in circulating cells of the immune system are often abnormally expressed in metastatic cells of solid tumors, thereby providing a mechanistic explanation for cancer progression. Fewer genetic or epigenetic changes are required for the development of liquid tumors than for solid tumors: this is one reason that epidemiologically liquid tumors reportedly need a shorter period of exposure to potent carcinogenic agents.

Inflammatory pathways that affect NF-kB, STAT3 and AP-1 are activated by tobacco, stress, dietary agents, obesity, alcohol, infectious agents, irradiation, and environmental stimuli, and in turn regulate expression of cytokines, adhesion molecules, proteases, anti-apoptotic factors, DNA repair factors, cell cycle and cell metabolism factors. NF-kB, STAT3 and AP-1 thereby control cell transformation, cell survival, proliferation, invasion, angiogenesis, metastasis, chemoresistance, and radioresistance of cancer. Deregulated activation of NF-kB, STAT3 and AP-1 family proteins is often associated with transition of cells from a differentiated phenotype to a stem cell-like unit, which may abnormally retain features of the differentiated cell. Common theme is the presence of features that are essential for the preservation of cell functions under unfavorable conditions related to stress,

The authors have no conflict of interest.

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Edited by: *Konstantinos Stathopoulos*

Accepted *7 January 2019*

both external and internal. Inflammatory mediators and neighboring cells affect stem cell function drastically.

At the same time besides inflammation, energy consumption and metabolism in cancer is considered to be of paramount importance towards the understanding of tumor mechanics. To our knowledge, there is not enough information available on the metabolic mechanisms underlying tumor cells. It might be bold to say, but not exaggerated, that a possible answer in the treatment of cancer, lies within the delicate mechanisms of metabolic processes. The governing idea was that the *Warburg effect* is rather the consequence of the cancer process, mainly due to hypoxic growth, and not to a prerequisite for cancer progression and proliferation *per se*[1]. However, it has recently been reported that the process of aerobic glycolysis is reversible from very simple compounds such *Dichloroacetate (DCA)*[2,3]. In actual fact, the aspect of interfering with cancer metabolic pathways as a mean for tumor treatment and therapy seems to be of utmost importance[4]. This became apparent from discoveries made in the field of metabolomics involving molecules previously thought to be solely metabolic and without the slightest suspicion for regulatory functions. Such molecules include diacylglycerol[5], ceramides and sphingosine, which are essential in cell proliferation and apoptosis[6] as well as pyridine nucleotides, which play an essential role in signal transduction[7].

Nowadays, it is clear, that metabolites or metabolic molecules, not only participate in metabolic processes which are dealing with the energy production and the thermodynamical conservation of the cell, but also possess multiple functions especially on the level of signal transduction. Consequently, similar molecules used, such as nutrients, could afford target therapy. Thus, in the present work we attempted to review some of the metabolic mechanisms of tumor cells focusing on the example of rhabdomyosarcoma.

Cancer metabolism

Cancer cells need greater amount of energy because they grow rapidly. *Otto Warburg* found that cancer cells prefer to produce ATP by glycolysis rather than by oxidative phosphorylation (OXPHOS) even in the presence of ample oxygen[8]. This finding was later termed the *Warburg* effect also known as "aerobic glycolysis". It is suggested that tumor cells preferentially used aerobic glycolysis over OXPHOS for glucose-dependent ATP production due to mitochondrial impairments[9]. Main characteristics of *Warburg* effect are an increased glucose uptake and synthesis of glycogen, lactate production and induced acidosis, which lead to an acid-mediated tumor invasion and further impairment of mitochondrial function in cancer cells[10].

Glucose transporters (GLUTs) sufficiency, glycogen synthase kinase-3 (GSK3) regulation, 6-phoshofructo-1-kinase (PFK-1) inhibition due to intracellular pH decrease, overproduction of reactive oxygen species (ROS), free radicals-which can initiate damage to macromolecules and to intracellular and extracellular signaling pathways-and the overexpression of a mitochondrial uncoupling protein has been shown to play important role in malignant onset, progression and immortality[8,11].

Studies using MCF10 cells (a normal human mammary epithelial cell line) suggested that the glucose flux through the Pentose Phosphate Pathway (PPP), in parallel to glycolysis, is also increased in cancer cells[12]. Increased PPP rate allows producing enough ribose for nucleotide synthesis and NADPH for lipid biosynthesis and maintenance of cell's redox status. Although glycolysis predominates as the main ATP supply, anti-glycolytic treatment in mature MCTs (multicellular tumor spheroids) has shown only a marginal effect on inhibiting tumor proliferation, suggesting that cells inside the spheroids remain significantly dependent on the alternative ATP derived from mitochondria[13].

To explain both the way ATP is produced via a low efficiency method, i.e. glycolysis, despite extremely high energy demand of the tumor cells, and reasonably explain the "autophagy paradox"[14], a new model is hypothesized: the reverse *Warburg* effect. According to this hypothesis, glycolysis occurs in mesenchymal stroma cells under the activation of neighboring cancer cells. Furthermore, an increased formation of recycled nutrients is produced. This high-energy metabolism is transferred to the neighboring cancer cells by the orientation of transport in the tricarboxylic acid (TCA) cycle. The consequence is that the OXPHOS increases enhancing ATP production, thus constituting metabolic coupling[8].

Going back to the *Warburg* effect, its establishment in cancer cells is the result of altered signaling pathways due to mutations that lead to the activation of growth promoting oncogenes and inactivation of tumor suppressors. Particularly, signaling alterations are found in: PI3K/AKT/mTOR pathway, RAS/RAF/MEK)/ERK kinase cascade, HIF-1 transcription factor, MYC transcription factor, AMPK signaling, P53 transcription factor[15].

In cancer, the PI3K/AKT/mTOR pathway reprograms glucose metabolism to fuel cell growth[15]. Aberrant activation of this pathway drives tumor progression independence on extrinsic growth factor stimulation through mutations in tumor suppression genes, such as *PTEN*, mutations in the components of the PI3K complex itself or by hyper-activation of RTKs[16]. Activated AKT stimulates glycolysis by phosphorylating key glycolytic enzymes, such as hexokinase 2 (HK2) and phosphofructokinase 2 (PFK2) which is an allosteric activator of PFK1[17]. AKT activation also increases the expression and membrane translocation of GLUTs in the cytoplasm for oxidation of glucose to pyruvate[18-20]. Finally activated AKT provide a strong signal toward its downstream element mTOR, which in turn directly stimulates anabolic processes and indirectly stimulates metabolism by activating key transcription factors such as c-MYC, cyclin D, HIF-1a even under normoxic condition[21].

Since cancer cells divide rapidly, their proliferative rate will always exceed the rate of new blood vessel formation. Therefore, they must, at some point experience a hypoxic condition which is anticipated by HIF-1 through transcriptional activation of many glycolytic genes. In summary, it could be said that HIF-1 activation results to: a) increased glucose import into tumor cells through the induction of enzymes involved in the overexpression of GLUT1 and GLUT3 and b) increased glycolysis rate through the induction of enzymes involved in the expression of glycolytic enzymes such as HK1/2 and pyruvate kinase M2 (PK-M2) and more[15]. HIF-1 activation also: c) inhibits OXPHOS by up-regulating genes such as pyruvate dehydrogenase kinase 1 (PDK1) and LDH-A[22,23] thus, preventing pyruvate entrance into the TCA cycle and making pyruvate available for conversion into lactate by LDH-A[23]. Activation of RAS/RAF/MEK)/ERK kinase cascade can also up-regulate the HIF-1a protein translation[18,20,24]. Activated ERK is further involved in stabilizing the binding of the active HIF-1a complex to RNA polymerase to positively modulate genes expression[19].

AMPK during period of energy stress (high AMP/ATP ratio) becomes activated and inhibits mTORC1, a downstream element of the PI3K pathway and consequently suppresses anabolic processes by blocking protein translation and fatty acid synthesis, being responsible for shifting cell to an OXPHOS. It is therefore clear that AMPK plays a major role in decreasing the growth of a rapidly dividing cell. Activation of AMPK is controlled by three upstream kinases, the tumor suppressor LKB1, calmodulin-dependent protein kinase kinase β and TAK1. Abnormalities in the AMPK signaling allow the activation of mTOR and HIF1, i.e. promote tumor progression.

It is also well known that elevated c-MYC and HIF-1a transcriptional activity along with insufficient P53-mediated control, allow up regulation of many glycolytic enzymes such as HK2, PFK1, TPI1, LDH-A, among others, in tumors. Moreover, c-MYC is responsible for the predominant production of PK-M2 over PK-M1 and also promotes the production of NADPH in order to match the increased ATP production and to support the rapidly dividing cells[15]. Tumor cell with mutated *p53* and loss of *TIGAR* expression causes increased glycolysis at the expense of the PPP, as well as increased ROS production[25]. Furthermore, loss of *p53* leads to reduced OXPHOS in the mitochondria thus forcing cells to depend on glycolysis for energy production[26].

Cancer cells need also to regulate *one-carbon metabolism* in a way it will provide them with the building blocks, as well as the reducing power, necessary to maintain high rates of proliferation. Proto-oncogenes with altered function, hijack sensing mechanisms to sustain one-carbon metabolism[27]. For example, *c-Myc* induces serine synthesis pathway (SSP) activity under nutrient deficient conditions in liver carcinomas[28]. As known, oncogenic mutations promoting constitutive KRAS activation, lead to aberrant activation of mTOR, which regulates both the SSP and the folate cycle[29,30]. In *KRAS* mutant tumors, loss of *AMPK* is prevalent as well[31], suggesting that along with stimulating mTOR, KRAS may also increase one-carbon metabolism through subversion of AMPK's inhibitory effect in the folate cycle[27]. The tumor suppressor *p53* is also known to play a key role in one-carbon metabolism. Particular, loss of *p53* causes addiction to serine and consequently, serine starvation has been shown to considerably decrease growth of these tumors[32].

Concluding, the *Warburg* effect allows that glucose can be used for macromolecule biosynthesis (through PPP and one-carbon metabolism) rather than for complete oxidation through the TCA cycle in the mitochondria. Nevertheless, TCA cycle continues to exist because glucose is partially substituted by glutamine (glutamine anapleurosis). Glutamine utilization via glutaminase (glutaminolysis) provides cancer cells with glutamate that, becoming in turn oxaloacetate, may enter the TCA cycle[33-36]. In addition to dependency on glutaminolysis, the majority of human cancers, express high levels of fatty acid synthase (FAS), a key metabolic enzyme that is functional to catalyze the synthesis of long chain saturated fatty acids for supporting the increased demand for membrane biogenesis[37,38].

Rhabdomyosarcoma metabolism

RMS tumors share several molecular signatures with common malignancies that would predict important metabolic adaptations. Molecular hallmarks having impact on RMS metabolism and driving metabolic changes in RMS are: Pax3-Foxo1 oncoprotein, deliberate activation of RTK pathways, loss of *p53* activity and oxidative stress pathways[39].

PAX3-FOXO1 transcription factor is a distinctive molecular signature generating from a chromosomal translocation[40,41]. The fused *Pax3-Foxo1* gene is found in 70% ARMS subtype cases (fusion-positive subset of RMS) and is considered a strong predictor of poor prognosis[42]. In the metabolic context, *Pax3-Foxo1* has been shown to drive transcription of *GLUT4* gene, thereby eliciting increased glucose uptake[43]. It also has the ability to drive the transcription of genes like fibroblast growth factor receptor 4 (*FGFR4*) and insulin-like growth factor 2 (*IGF-2*) as well as the ability to elicit downregulation of *PTEN* gene[39]. Thus, fusion-positive RMS' are characterized by sustained activation of the RTK/RAS/PI3K/AKT/mTOR pathway; in addition these tumors often carry high copy number of *N-Myc* gene and exhibit IGF-2 overexpression due to loss of imprinting (LOI) at 11p15.5 locus[39]. The activity of AKT in ARMS cells with a Pax3-Foxo1-dependent transcription of the carnitine palmitoyltransferase gene (CPT1A), the outer mitochondrial enzyme responsible for the formation of acyl carnitines, was reported to facilitate lipid degradation[44]. This mechanism has been supposed to provide ARMS cells with the burst of energy required to migrate and metastasize[44]. Both fusion-positive and –negative RMS subsets share a deliberate activation of RTK-dependent pathways[45]. Different lines of evidence indicate that the PI3K/AKT and

ERK1/2 pathways, often concurrently with the HIF pathway, play a central role for RMS progression[39].

A gene signature consistent with the "*p53* off" state has been significantly found in fusion-negative RMS with an incidence from 5% to 19%[45,46], whereas to a lesser extent in fusion-positive tumors[46,47]. Besides glucose consumption, further experimental evidence is required to address the question of whether *p53* loss may impact other key metabolic pathways in RMS[39].

A large number of genetic anomalies, particular RAS pathway mutations, have been identified in ERMS[46] but not in ARMS[48]. RAS-positive ERMS cells acquire specific characteristics, e.g. higher rates of G to T transversions, due to mutations caused by oxidative damage. Moreover, ERMS displayed methylation of several genes implicated in regulation of metabolism, mitochondrial function, and oxidative stress, including *PTK2, COX7A1, NOS1P, NOS1, ATP2A3, DDAH1, GLRX,* and *TXNDC12*[46]. Hence, ERMS tumors may benefit of increased ROS levels for cell growth, but can also progressively accumulate ROS-induced gene mutations making them susceptible to cell death. Endogenous levels of *SPRY1* (*Sproutyl 1*), which is an upstream antagonist of RAS that is activated by extracellular signal-related kinase (ERK), seem to protect oncogenic RAS-positive ERMS cells from the induction of cell death[48]. Thus, silencing of *SPRY1* triggers complete regression of RMS tumors carrying *RAS* gene[48].

Cancer specific energy metabolism in RMS, particular in ARMS cells is regulated by microRNA[49]. It is demonstrated that the expression levels of muscle-specific miR-1 and miR-133b were downregulated in RMS cells and that the ectopic expression of miR-1 and miR-133b in RMS cells showed an antitumor effect by inducing autophagic cell death through silencing of *polypyrimidine tract-binding protein 1 (PTBP1)*, a splicer of pyruvate kinase muscle (PKM) mRNA and positive regulator of cancer-specific energy metabolism. Moreover, the knockdown of *PAX3-FOXO1* reduced the expression of *PTBP-1*, resulting to the PKM1-dominant expression instead of PKM2[49], which is defective in catalyzing pyruvate production[50], so alternative substrates must be oxidized to generate ATP[51-53]. One possibility is to oxidize glutamine/glutamate to produce pyruvate, bypassing the defective PK step, and allowing lactate production to regenerate NAD^+ [50,52,54].

Bioenergetic properties and metabolic vulnerabilities

In a recent study[55], by applying stable isotope tracing methods, using [U-^{13}C]-glucose as tracer and isotopomer-based metabolomics analysis, it is demonstrated that there are major distinctions in central energy and anabolic metabolism between the primary myocyte and transformed Rh30 (ERMS) cell lines (RMS cells showed an energy producing anabolic metabolic phenotype). Particular, the data showed that relative to the myocytes, glycolysis, *Krebs'* cycle, pentose phosphate pathway, and nucleotide biosynthesis were coordinately enhanced in Rh30 cells, presumably to meet the demand of accelerated growth. The coordination between energy production (including the *Warburg* effect) and generation of biosynthetic precursors in Rh30 cells may well be mediated via an activation of anaplerotic carboxylation and the malate/Asp shuttle which facilitates the transfer of NADH hydride from the cytoplasm to the mitochondrion for oxidative phosphorylation, thereby sustaining accelerated glycolysis[55]. It is therefore evident that mitochondria of transformed Rh30 cells are active, both in citric acid cycling and respiratory electron transport, processes that are essential to cell proliferation[55].

Nevertheless, in another study[56], evidenced that there is mitochondrial respiratory deficiency in R1H cells. Particular, a marked decrease in the cardiolipin content of R1H cells cultured in growth and differentiation media, together with a significant increase in the content of mitochondrial biogenesis factors and mitochondrial respiratory chain proteins, were evidenced. These data indicate that the mitochondrial inner membrane composition and the overall process of mitochondrial biogenesis are markedly altered in R1H cells[56]. Importantly, the dysregulation of protein-to-cardiolipin ratio was associated with major deficiencies in both basal and maximal mitochondrial respiration rates. This deficiency in mitochondrial respiration probably contributes to the inability of R1H cells to decrease mitochondrial H_2O_2 level at the onset of differentiation[56]. Further, it is showed that in R1H not only the amount of mitochondria was decreased, but also those tumor mitochondria present specific functional properties[57]. More specific, functional abnormalities of the respiratory chain complexes were found, in contrast to muscle cells. In R1H a decrease of complex I-activity and a varied ratio of pyruvate-dependent succinate respiration in favor of succinate respiration were verified[57].

Data suggest that ARMS cells may be more dependent on glycolysis than mitochondrial respiration for ATP production. The increased dependency of ARMS cells on glycolysis is consistent with 2-DG-induced growth inhibition results showed in the same study[58]. It is also known that ARMS cells are more sensitive than ERMS cells to 2-DG-induced apoptosis[59]. Furthermore, it is showed that 2-DG-induced growth inhibition is inversely related to respiration rates and glycolytic reserve capacity, suggesting that differences in cellular bioenergetics may explain, at least in part, the differences in 2-DG sensitivity between ARMS and ERMS cells, as well as the relative insensitivity of osteosarcomas to 2-DG[58]. Lately, it was found that 2-DG promotes endoplasmic reticulum (ER) stress and induces classical nuclear apoptotic morphology and caspase-dependent cell death mediated by ATF4[60]. In fact, 2-DG interferes with N-glycosylation, an effect that can be prevented by the hexose mannose but not glucose. When glycosylation is impaired, improper folding of proteins in the ER activates the protein kinase RNA-like endoplasmic reticulum kinase (PERK) that phosphorylates eIF2a, thus inhibiting translation of most proteins in the

cell. One of the exceptions is ATF4/cAMP-response element binding protein 2, a transcription factor that is selectively translated under ER stress. ATF4, which regulate multiple genes aimed at restoring homeostasis in the ER, may end up killing the cell by activating mitochondrial or death receptor-induced apoptosis, an effect that is mediated in many cases by the transcription factor C/EBP homologous protein (CHOP)/Gad-dl53[60].

Similar to 2-DG, glucose deprivation (GD) impairs glycosylation, as well glycolysis. Moreover, both conditions should affect glucose flux through the pentose phosphate shunt and, consequently, its function. GD deprivation -a common occurrence during tumor development- can promote severe bioenergetic stress leading to cell death. In cancer cells, impairment of glycolysis and subsequent ATP loss is considered to be the most important effect of GD leading to cell death[60].

Perturbations at specific points in the metabolic network can induce muscle myoblasts to differentiate into myosin heavy chain-expressing myotubes[61]. In other words, carbon metabolism is connected to the cellular differentiation network and can provide a novel method for controlling myogenic differentiation. Using RNAi screening, metabolic profiling and small-molecule probes, three metabolic enzymes whose knockdown induces differentiation of mouse C2C12 myoblasts even in the presence mitogens where discovered: phosphoglycerate kinase (Pgk1), hexose-6-phosphate dehydrogenase (H6PD) and ATP citrate lyase (Acl)[61]. Similar, it is shown that this finding can be applied to small molecule-based differentiation of human RMS. Treatment of the human RD ERMS cell line with fluvastatin induced RMS cell differentiation, reduced cancer cell proliferation and inhibited anchorage-independent growth[61]. Myogenic differentiation and cellular metabolism are coordinated via at least three cellular pathways: calcium/calcineurin signaling, chromatin acetylation and the cholesterol biosynthetic pathway. It is suggested that metabolic enzymes may be important for the control of cancer cell growth and differentiation, and that metabolic perturbations may result in important changes in cell states. Alterations in the metabolic network may serve as novel therapeutic approaches to muscle sarcoma differentiation therapy[61].

Targeting of the molecular network that support sarcoma cell proliferation identified actionable gene targets encoding enzymes relevant to amino acid biosynthesis: asparagine synthetase (ASNS), branched chain aminotransferase 1 (BCAT1), phosphoserine aminotransferase (PSAT1) and phosphoglycerate dehydrogenase (PHGDH)[62]. Silencing of ASNS, an amidotransferase that converts aspartate into asparagine, produced the strongest inhibitory effect on sarcoma growth in a functional genomic screen of mouse sarcomas generated by oncogenic *KRAS* and disruption of *Cdkn2A. ASNS* silencing in mouse and human sarcoma cell lines reduced the percentage of S phase cells and impeded new polypeptide synthesis. These effects of ASNS silencing were reversed by exogenous supplementation with asparagine. Asparagine depletion via the ASNS inhibitor amino sulfoximine 5 (AS5) or asparaginase, inhibited mouse and human sarcoma growth *in vitro*, and genetic silencing of ASNS in mouse sarcoma cells combined with depletion of plasma asparagine inhibited tumor growth *in vivo*, as well[62]. A recent report demonstrated that asparagine can suppress cell death in glutamine-deprived cells[63], implicating asparagine in promoting cellular adaptation to metabolic stresses such as glutamine depletion. This study also noted that asparagine is the last amino acid synthesized in the TCA cycle and that its animation depends exclusively on glutamine[63]. Amino acid availability is known to stimulate mechanistic target of rapamycin (mTOR) complex 1, which integrates environmental and intracellular signals to regulate cell growth[64]. Taken together, these observations suggest that asparagine may serve a central role as a cellular sensor of TCA cycle intermediate/ reduced nitrogen availability and, ultimately, as a metabolic regulator of cell behavior. Asparagine reliance on sarcoma cells may represent a metabolic vulnerability with potential anti-sarcoma therapeutic value[62].

Signaling pathways driving neoplasia related to RMS metabolism

Solid tumors often contain hypoxic microenvironments due to abnormal vasculatures and outweighing demands of oxygen[65]. As already told, cancer cells rely on anaerobic respiration, leading to sequential acidic microenvironments, mainly attributed to excessive protons caused by overexpressed M2-PK (M2-pyruvate kinase, pyruvate kinase dimer isoenzyme)[66], as well. Hypoxic and acidic microenvironments cause genetic instability and activate signaling pathways, contributing to cancer progression and resistance[65].

Hypoxia stimulates multiple pathways by upregulating and activating Hypoxia Inducible Factors (HIFs)[67] which, are the master driving forces of the cellular adaption to hypoxia[68]. In turn, HIFs upregulate the expression of Vascular Endothelial Growth Factor (VEGF)[69], GLUTs and Lactate Dehydrogenase (LDH), key proteins in tumor glycolysis cascade and bioenergetics[70]. It is known that HIF-2α activates TGF-α/ EGFR signaling pathway in hypoxic Hepatocellular Cancer Cells (HCC) cells[71]. In addition, hypoxia activates autophagy through HIF-1α, PKC, JNC and mTOR pathways[72]. HIF-1α also activates HGF/c-MET signaling pathway that relaxes the adhesion between tumor cells and degrades the extracellular matrix, thereby enhances the invasion and metastasis of tumor cells[73].

Lactic acid (produced in large amounts due to *Warburg* effect in cancer cells[74]), is transported into tumor microenvironment by Monocarboxylic Acid Transporter (MCT)[75]. At this point it should be noticed that protein kinase δ (PKCδ) and HIF-1α can be involved in the up-regulation of MCT4 expression in RD cells[76].Once entering endothelial

cells, lactic acid causes degradation and phosphorylation of IκBα (inhibitor of NF-κB), stimulating cell migration and blood vessel formation through the NF-κB/IL-8 pathway[77]. Inhibition of classical NF-κB activity in sarcoma cell lines restored alternative signaling (turned off Ras/Raf or Akt-mediated pathways and HIF activity) as well as an increased oxidative respiratory metabolic phenotype *in vitro*[78]. In addition, microarray analysis indicated that inhibition of NF-κB in sarcoma cells reduced glycolysis[78].As conclusion, it is said that classical NF-κB metabolically reprograms sarcoma cells through regulation of hexokinase 2[78].

In a review related to novel pathways and molecular targets for the treatment of sarcoma[79] it is referred that expression of *PAX3-FOXO1* (in more than 75% of ARMS) is thought to halt normal muscle differentiation by several mechanisms, including suppression of *MyoD* and the activation of cyclin D1/cyclin-dependent kinase 4 (CDK4) complexes.

In RMS, it is clear that the hedgehog (Hh) pathway activation (ligand-independent due to mutation in pathway genes[80] or ligand-dependent involving autocrine or paracrine signaling[81]) contributes to tumorigenesis at multiple stages – initiation, growth, maintenance, metastasis, and recurrence[82]. However, Hh role in each is often confounded by signals from other pathways[82]. Crosstalk between aberrantly activated Hh and other actionable pathways -RAS/RAF/MEK/ERK, PI3K/mTOR, EGFR, and Notch- is evidenced in RMS and summarized in a review article[82].

It is shown that MEK/ERK inhibitor U0126 dramatically prevented rhabdoshpere (RD) formation (ERMS cell line) and down-regulated stem cell (responsible for initiation, propagation, invasiveness and metastasis) markers CD133, CXR4 and *Nanog* expression, but enhanced ALDH, MAPK phospho-active p38 and differentiative myogenic markers. The RH30 cell line did not exhibit persistent ERK inhibition by U0126 whereas it did exhibit very low levels of CD133, thus suggesting that other pathways underlie the ARMS cell-like phenotype[83]. MEK/ERK pathway is sustaining tumorigenicity and *in vitro* radioresistance of ERMS stem-like cell population[83] probably inducing the expression of the cell cycle inhibitor $p21^{WAF1}$ [84].

MET (a receptor of hepatocyte growth factor, HGF), is reported to be downstream of the *PAX3-FOXO1* fusion gene specific to ARMS, and a key mediator of metastatic behavior in RMS[85]. Data from a recent study[85] suggest that HGF/MET signaling promotes motility of ARMS cells mainly through ERK2 signaling.

Inhibiting mTOR pathway with rapamycin induces apoptosis of Rh1 cancer cells and it is shown that growth factors have different effect on rapamycin induced apoptosis of Rh1 cells: IGF-1 and insulin totally prevented apoptosis but in contrast EGF and PDGF showed only marginal effect on viability[86]. Having established conditions for prolonged inhibition of MEK1 signaling, it was determined that activation of Erk1 and Erk2 is not required for IGF-1 mediated protection from rapamycin-induced apoptosis. Similar, it is shown that IGF-1 mediated protection of Rh1 cells from rapamycin-induced apoptosis is independent of Akt signaling.[86] Although there are various levels of crosstalk between the MAPK and the PI3K/Akt pathways, which may play a role in regulating apoptosis, it was shown that neither the MAPK nor PI3K/Akt-dependent pathways are required for IGF-1 to protect against rapamycin-induced apoptosis, suggesting that a novel pathway(s) is responsible for the IGF-1 mediated protection in these RMS cells[86].

IGFBPs 1-6 play an important role in modulating IGF activities but some IGFBPs may have an IGF-independent effect, including induction of apoptosis and modulation of cell migration[87]. It is shown that p38 MAPK is involved in this IGFBP-6-induced IGF-independent RD cell migration[87]. Cross-talk between MAP kinase (ERK1/2, p38 and JNK MAPKs) pathways is involved in IGF-1 independent, IGFBP-6-induced Rh30 cell migration, as well[88]. In addition, TPA/PKCα-mediated ERK, JNK and p38 activation regulates the myogenic program in human RMS cells[89]. Data also indicate the existence of a network formed by myostatin/SMAD2/3, ERK and p38 pathways that, when deregulated, might contribute to the pathogenesis of ERMS[90]. In another study[91], it is demonstrated that p38 MAPK, CaMK-, and calcineurin-mediated signaling pathways transcriptionally regulate myogenin expression.

Activation of RAGE by its ligand, HMGB1, stimulates myogenesis via Cdc42-Rac1-MKK6-p38 mitogen activated protein kinase pathway. In addition, inactivation of RAGE in myoblasts results in reduced myogenesis, increased proliferation, and tumor formation *in vivo*[92]. Human RMS cells highly express a tissue factor that promotes thrombin formation which activates platelets to generate microvesicles (PMVs), which transfer to RMS cells α2β3 integrin and increase their adhesiveness to endothelial cells. Accordingly, RMS cells covered with PMVs showed higher metastatic potential. Furthermore, PMVs activate ERK1/2 and Akt to chemoattract RMS cells. It is also found that RMS cells express functional protease-activated receptor-1 (PAR1) and PAR3 and respond to thrombin stimulation ERK1/2 and MAPK p38 phosphorylation[93].

Growth of Kym-1 RMS cells depends on endogenous receptor tyrosine kinase signals activated by insulin and IGF, as revealed from enhancement of proliferation by insulin and IGF-1 and cytostatic action of inhibitors of IR/IGFR kinases. Depending on the presence or absence of caspase inhibitor z-VAD-fmk, TNF induced full growth arrest or apoptosis, respectively, indicating dominance of TNF over mitogenic signal pathways in Kym-1 cells. In accordance with a caspase-independent cytostatic action, TNF downregulated IR kinase activity and caused a profound inhibition of downstream mitogenic signals including the MAPK cascade and STAT5, key pathways of proliferation and cell survival[94]. It is also shown that persistent GP130/STAT3 signaling contributed to resistance of anti-cancer drug treatments in RMS cells[95].

Finally, a computational approach, using cDNA microarrays, in order to identify commonalities between T-cell acute lymphoblastic leukemia (CCRF-CEM) and the embryonal rhabdomyosarcoma (TE-671) cell line predicted that JAK1, STAT1, PIAS2 and CDK4 are the driving forces that transform physiological cells to malignant[96].

Conclusions

Mutations that lead to the activation of growth promoting oncogenes and inactivation of tumor suppressors alter signaling of PI3K/AKT/mTOR pathway, RAS/RAF/MEK)/ERK kinase cascade, HIF-1 transcription factor, MYC transcription factor, AMPK signaling and P53 transcription factor. Among all signaling alterations, prominent in cancer is the signaling alteration of PI3K/AKT/mTOR pathway because it reprograms glucose metabolism to fuel cell growth. Proto-oncogenes with altered function hijack sensing mechanisms to sustain one-carbon metabolism in order to provide them with the building blocks, as well as the reducing power, necessary to maintain high rates of proliferation. Although glycolysis predominates, TCA cycle continues to exist in cancer cells because glucose is partially substituted by glutamine. RMS metabolism follows pattern of cancer metabolism with Pax3-Foxo1 oncoprotein, deliberate activation of RTK pathways, loss of *p53* activity and oxidative stress pathways being drivers of metabolic changes in tumor cells. Hypoxic and acidic microenvironments in solid tumors cause genetic instability and activate signaling pathways, contributing to cancer progression and resistance. Signaling pathways driving neoplasia in RMS are mainly Ras/Raf/MEK/MAPK (i.e. ERK1/2, p38 and JNK), MET/ERK, HGF/MET, Hh, STAT and last but not least PI3K/Akt/mTOR. These pathways regulate plethora of transcriptional factors which play important role in RMS metabolism and all stages of RMS tumorigenesis.

Authors' contributions

CT: collected literature, drafted the manuscript, GIL: proof-read the manuscript, drafted the manuscript, provided critical review, gave final permission for submission.

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