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Review

Re-evaluating the role of FOXOs in cancer

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ABSTRACT

FOXO transcription factors are negatively regulated by the PI3K-PKB/AKT signaling pathway and have been mainly considered to be tumor suppressors due to their inhibitory effect on cancer cell growth and survival. However, FOXOs can also support tumor development and progression by maintaining cellular homeostasis, facilitating metastasis and inducing therapy resistance. In agreement with these opposing views on the role of FOXOs in cancer, studies using FOXO levels or activity as prognostic markers for cancer patient disease progression and survival came to contradicting results. While it is clear that FOXOs are involved in various aspects of cancer, it is debatable whether FOXOs function as tumor suppressors or supporters, or may be both depending on the context. In this review, we describe the role of FOXOs in signaling pathways and processes relevant to cancer and evaluate recent advances in understanding the role of FOXOs in cancer. Based on recent insights it becomes clear that FOXOs may not be classical tumor suppressors and that targeting FOXO activity might hold promise in cancer therapy.

1. Introduction

For a normal cell to become a cancer cell it has to obtain various traits, summarized as the hallmarks of cancer [1]. Two of the Hallmarks a potential tumor cell needs to acquire are “sustained proliferative signaling” and “evading growth suppression”. Acquisition of sustained proliferative signaling commonly arises from alteration in components of growth factor receptor (GFR) signaling pathways. Mutations that are commonly found in PI3K-PKB/AKT pathway members commonly underpin oncogenic cell proliferation and survival [2]. Key transcription factors negatively regulated downstream of PI3K-PKB/AKT signaling are members of the Forkhead Box O family (FOXO). FOXOs have been put forth as putative tumor suppressors that need to be inactivated in order to evade growth suppression based on tissue culture experiments showing their cytostatic and apoptotic potential. They are involved in a plethora of cellular functions that control many different aspects of life including lifespan, diabetes and cancer [3]. In this review we describe the role of FOXOs in cancer and evaluate recent advances in this field. By doing so we unveil that FOXO is not merely a classic tumor suppressor and illustrate a more complex supportive role for FOXOs in cancer.

Abbreviations: RTK, receptor tyrosine kinase; GF, growth factor; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; PKB, protein kinase B; AKT, RAC-alpha serine/threonine-protein kinase; FOXO, forkhead box O; GFR, growth factor receptor; PDPK1, Phosphoinositide-dependent kinase-1; PTEN, Phosphatase and tensin homolog; SMAD, Mothers against decapentaplegic homolog 4; STAT, Signal transducer and activator of transcription; CBP, CREB-binding protein; HDAC, Histone deacetylase; SIRT, NAD-dependent deacetylase sirtuin; MDM, Mouse double minute; SESN, sestrin; SOD, super oxide dismutase; GPX, glutathione peroxidase; PRMT, Protein arginine methyltransferase; RUNX, Runt-related transcription factor; ERK, extracellular signal-regulated kinase; MST1, mammalian sterile 20–like kinase; NFKB, nuclear factor kappa-light-chain-enhancer of activated B cells; IKKα/β, I-kappaB kinase; CDK, cyclin dependent kinase; SGK, serum and glucocorticoid-regulated kinase; DYRK1, Dual specificity tyrosine-phosphorylation-regulated kinase; CK1, casein kinase; CKI, cell cycle kinase inhibitor; mTORC, mammalian target of rapamicin complex; PIP3, Phosphatidylinositol (3,4,5)-trisphosphate; IPO, Importin; TNPO, transportin; ASK, Apoptosis signal-regulating kinase; JNK, c-jun terminal kinase; ATM, ataxia-telangiectasia mutated kinase; ROS, reactive oxygen species; CAT, catalase; IRS, insulin receptor substrate; IGFBP, insulin-like growth factor receptor binding protein; AMPK, AMP-activated protein kinase; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; FAS, Fas cell surface death receptor; DR, death receptor; BAK, Bcl-2 homologous antagonist/killer; BAX, BCL2-associated X; BCL2, B-cell lymphoma 2; BCL-XL, B-cell lymphoma-extra large; MCL, induced myeloid leukemia cell differentiation protein; BH3, BCL2 homology domain 3; BIM, Bcl-2-like protein 11; BAD, Bcl-2-associated death promoter; BMF, Bcl-2-modifying factor; BID, BH3 interacting-domain death agonist; PUMA, p53 upregulated modulator of apoptosis; NOXA, Phorbol-12-myristate-13-acetate-induced protein; BOK, Bcl-2 related ovarian killer; ER, estrogen receptor; PAX, paired box; MLL, myeloid/lymphoid or mixed-lineage leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; MMP, matrix metalloproteinase; BCR-ABL, B-cell receptor - abelson murine leukemia viral oncogene homolog; LIC, leukemia inistiating cell; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; ERBB, erb-b2 receptor tyrosine kinase; RET, rearranged during transfection; RAS, rat sarcoma viral oncogene homolog; MEK, Mitogen-activated protein kinase; IQGAP, IQ motif containing GTPase activating protein; MYC, myelocytomatosis viral oncogene homolog

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1.1. Canonical FOXO regulation

The mammalian Forkhead box O (FOXO) family of transcription factors consists of four family members; FOXO1, FOXO3, FOXO4 & FOXO6, which are evolutionary conserved and function as transcription factors through binding to the DNA consensus sequence 5′-TTGTT TAC-3′ [3–5].

The PI3K-PKB/AKT pathway negatively regulates transcriptional activity of FOXO1, FOXO3 & FOXO4. As the regulation of FOXO6 is less dependent on canonical PI3K-PKB/AKT signaling and because FOXO6 expression seems limited to the central nervous system, liver, kidney cortex and stomach, and because this is the least studied FOXO family member we mainly focus on FOXO1, FOXO3 & FOXO4 (FOXOs) in this review [6]. Upon activation of growth factor receptor tyrosine kinases, PI3K becomes activated and generates PIP3 at the plasma membrane. PIP3 functions as a docking site for PDPK1 and PKB/AKT. PDPK1 activates PKB/AKT, which subsequently phosphorylates a wide array of target proteins to stimulate glucose uptake, cell proliferation and survival [7].

Transcriptional activity of FOXOs is regulated through shuttling between the nucleus and the cytoplasm. Phosphorylation of nuclear FOXOs by PKB/AKT at three conserved RxRxxS/T residues induces the binding to 14-3-3 proteins, which facilitate nuclear export of FOXO1, FOXO3 & FOXO4 and simultaneously obstruct relocation into the nucleus [8–10]. Upon loss of GFR signaling, net dephosphorylation of PIP3 by PTEN results in reduced PKB/AKT activity, loss of FOXO phosphorylation and subsequent nuclear accumulation of FOXOs. In the nucleus, FOXOs mediate transcription of a wide array of target genes involved in cell cycle inhibition, apoptosis, redox homeostasis, metabolism and angiogenesis (Fig. 1) [3,11].

It remains a point of debate whether FOXOs regulate a specific set of target genes or are more general activators of gene expression [12]. Regardless, upon accumulation in the nucleus, FOXOs preferably bind DNA in promoters and enhancers covered with histone marks correlated with active transcription [13,14]. These observations implicate that the output of FOXOs is heavily influenced by the epigenetic status of the DNA at the moment FOXOs reside in the nucleus. Additionally, several proteins involved in transcription regulation have been reported to functionally interact with FOXOs e.g. p300/CPB, β-catenin, PPARγ, estrogen receptor, androgen receptor, SMADs, STATs and RUNX [15].

1.2. FOXO activation by cellular stresses

Upstream regulation of FOXOs is not limited to RTK signaling. Especially in cancer, localization of FOXOs can be modulated through multiple other pathways. When a cell encounters stress like elevated reactive oxygen species (ROS) levels, nutrient starvation or DNA damage, FOXOs will be activated in order to partake in re-establishing cellular homeostasis [16].

When levels of ROS are high or the reductive capacity of the cell is low, FOXOs translocate to the nucleus in two different ways. First, JNK becomes activated in response to increased levels of ROS in the cell. This can occur through the redox sensitive kinase ASK1 or the small GTPase Ral. Activated JNK antagonizes RTK signaling by phosphorylation of the insulin receptor substrate adaptor proteins IRS1/2 thereby preventing GF signaling dependent inactivation of FOXO. JNK also phosphorylates FOXOs and 14-3-3 directly, stimulating nuclear translocation of FOXOs by preventing FOXO binding to 14-3-3 [17–20].

Second, under more oxidizing conditions in the cell cysteines in FOXOs can form disulfide bridges with nuclear importers TNPO1, IPO7 and IPO8, and subsequently translocate to the nucleus [21,22]. To counteract elevated ROS production in the cell, FOXO mediates the transcription of antioxidant genes like *CAT, SESN1/2/3, SOD2, PRDX3, GPX1, GSTM1* and genes involved in the metabolic generation of the Glutathione antioxidant system and reductive entities like NADPH [23,24].

Under conditions in which glucose is limited, ATP levels in the cell drop and the ATP/AMP sensor AMP kinase (AMPK) becomes active. AMPK phosphorylates FOXOs at Ser413, Ser588 and Ser626 (numbering of human FOXO3), resulting in nuclear localization and the stimulation of target genes involved in metabolic rewiring and stress resistance [25]. Next to the well-studied regulation of FOXOs by PKB/AKT, JNK and AMPK, many other kinases attenuate FOXO activity. In response to DNA damage FOXOs bind to and become phosphorylated by ATM kinase and hereby contribute to the DNA damage response and regulation of apoptosis [26,27]. FOXOs have also been described as targets of ERK, MST1, CDKs, SGK, DYRK1A, IKKα/β and CK1 [28]. To what extent these phosphorylation events influence FOXO activity and output is however still under investigation.

FOXOs can also be modulated through other site-specific modifications including acetylation, ubiquitination, methylation, PARylation, hydroxylation and glycosylation. Best studied are acetylation by actetyl transferase p300, and deacetylating enzymes such as HDACs and SIRTs, as well as ubiquitylation by MDM2 and USP7 or methylation by PRMT and SET9 (Fig. 1) [29–34]

Taken together FOXOs are regulated by many different upstream signals and, when activated, regulate the transcription of various genes. Due to this great complexity, it is no surprise that the current understanding of how FOXOs function exactly is still incomplete. As FOXOs function at the crossroad of diabetes, cancer and aging, it is essential to understand its functions in detail. Exemplary is the fact that after two decades of research it is still not clear whether FOXOs function as tumor suppressors or supporters [35].

2. The archetype: FOXOs are tumor suppressors

2.1. Repressing the cell cycle

It was already apparent in the first seminal papers that identified that FOXOs were negatively regulated downstream of PKB/AKT signaling, that FOXOs could have tumor suppressive functions. Activation of FOXOs, either by pharmacological inhibition of PI3K-PKB/AKT or the ectopic overexpression of FOXO resulted in a robust cell cycle arrest in fibroblasts and cancer cell lines derived from colon carcinoma, glioblastoma, osteosarcoma and acute T cell leukemia [8,9,36,37].

Cell proliferation starts from a quiescent state also known as G_0 and continues by progression from G_1 to S phase. In early G_1, expression levels of Cyclin D proteins (*CCND1/2/3*) are upregulated by GFR signaling, leading to increased levels of CyclinD-CDK4/6 complexes. Cyclin D-CDK4/6 complexes inhibit the retinoblastoma family of proteins (RB, p107 and p130), resulting in the release E2F transcription factors that induce transcription of S-phase proteins including Cyclins [38]. From S-phase onward, Cyclin-E/A-Cdk2 complexes take over to ensure correct DNA replication and cellular growth before entering mitosis. Once this process is completed, Cyclin B/Cdk1 complexes become active and mitosis starts.

FOXO-induced cell cycle arrest is mediated through transcription of multiple cell cycle kinase inhibitors (CKI). The best-described CKI downstream of FOXO is $p27^{kip1}$ (CDKN1B). Besides CDKN1B, FOXOs have also been described as regulators of $p21^{cip1}$ (CDKN1A), $p57^{kip2}$ (CDKN1C) and the INK4 family of CKIs, $p15^{INK4b}$ (CDKN2B), $p16^{INK4a}$ (CDKN2A), $P18^{INK4c}$ (CDKN2C) and $p19^{INK4d}$ (CDKN2D) [36,39–41]. FOXO-mediated induction of CKI expression leads to inhibition of the Cyclin/CDK complexes responsible for progression through the different phases of the cell cycle and results in a robust cell cycle arrest in G_0/G_1, G_2, or even senescence.

2.2. Stimulating apoptosis

Next to functioning as repressors of the cell cycle, FOXOs are well described as inducers of apoptosis in many different cell types [42]. Apoptosis can be triggered through multiple cell intrinsic and extrinsic

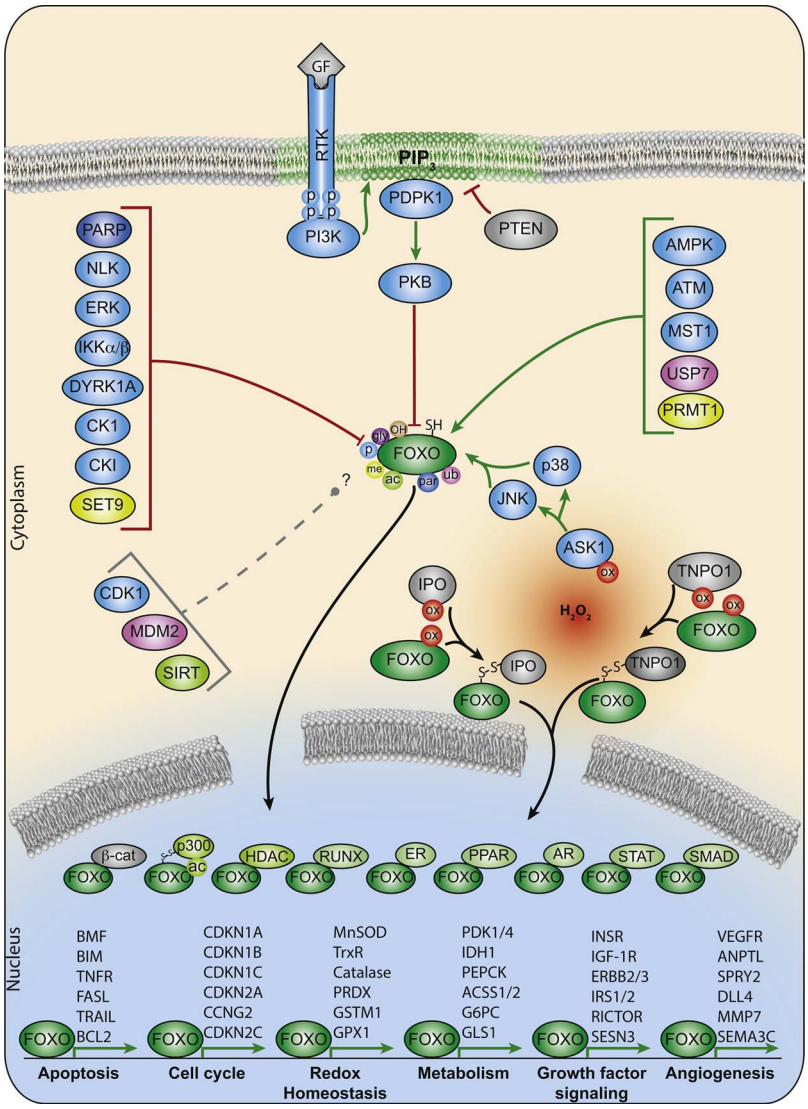

Fig 1. Regulation of FOXO transcription factors.
The PI3K-PKB/AKT pathway is the canonical pathway regulating transcriptional activity of FOXOs. Additionally, FOXOs can be modulated by multiple other kinases through phosphorylation (blue). Other post-translational modifications influencing FOXO activity include methylation (yellow), ubiquitination (pink), acetylation (light green) PARylation (dark blue), glycosylation (purple) and hydroxylation (beige). Upon accumulation in the nucleus FOXOs can bind various transcription-cofactors (green) and regulate the transcription of genes involved in the cell cycle, apoptosis, metabolism, redox homeostasis, GFR signaling and angiogenesis.

signals that converge on Caspase 3 and Caspase 7, which are the final and irreversible executioners of programmed cell death. Extrinsic apoptotic stimuli comprise of Tumor necrosis factor (TNF), TNF related apoptosis inducing- (TRAIL) and FAS-ligands (FasL) that activate the TNF (TNFR) and Death (DR) receptor family of extracellular receptors respectively [43,44].

The decision to undergo intrinsic apoptosis is determined by the status of the BAK and BAX proteins. In viable cells, BAK/BAX are in an inactive conformation on the outer mitochondrial membrane. Their activity is regulated through competitive binding of BAX and BAK between anti-apoptotic factors BCL2, BCL-XL or MCL1 and pro-apoptotic BH3-only proteins BIM, BAD, BID, PUMA, NOXA, BOK and BMF. Anti-apoptotic factors render BAK/BAX inactive, but upon binding to pro-apoptotic proteins BAK/BAX become active, leading to mitochondrial outer membrane permeabilization and apoptosis [45]. FOXOs can drive the expression of both extrinsic and intrinsic pro-apoptotic genes including: *FASL, TRAIL, TNFR, BIM* and *BMF*, leading to cell death in both normal and cancer cells [42].

FOXOs have recently also been described to induce anoikis: a form of apoptosis triggered by detachment from the extracellular matrix. Detachment from a tissue results in reduced GFR signaling and metabolic stress due to impaired glucose uptake. Under these conditions FOXOs drive the expression of *BMF* in order to execute anoikis and prevent metastasis [46,47].

2.3. FOXO loss enhances tumorigenesis in mice

Knockout studies unveiled that loss of *Foxo1* leads to lethality due to defects in angiogenesis in the mouse embryo [48,49]. *Foxo3* knockout is not embryonic lethal, but female mice become infertile after 15 weeks due to premature primordial follicle activation and subsequent depletion [50]. Loss of *Foxo4* did not result in any notable changes in the mouse [49]. Together, these studies described roles for FOXOs in development but no relation to cancer was encountered. The seminal paper of Paik and colleagues in which *Mx-Cre* inducible conditional *Foxo1/Foxo3/Foxo4* triple knockout mice were generated showed that FOXOs are redundant with respect to tumor suppression [51]. Only a mild increase in tumor incidence in aged mice was observed when two FOXO knockouts were combined e.g. *Foxo1/Foxo3* or *Foxo1/Foxo4*. But triple knockout induced in adult mice resulted in lymphoblastic thymic lymphomas in the spleen, liver and lymphatic system. Additionally, hemangiomas were detected and widely spread throughout the mouse. This study therefore solidified that individual FOXOs are indeed redundant and can function as tumor suppressors [52]. Curiously, the types of tumors that are found are only derived from the mesodermal lineages. This argues against a general role for FOXOs in prevention of tumor development in human cancer.

The importance of FOXOs as tumor suppressors could however be limited or obscured due to the presence of other tumor suppressors.

Also, to what extent FOXO6 can influence the observed tumor spectrum in Foxo1/Foxo3/Foxo4 triple knockout mice has not been reported to date. It might however be an interesting follow-up as it becomes more apparent that FOXO6 could be more widely expressed than previously reported [53]. Loss of FOXO broadens the tumor spectrum in $p53^{-/-}$ mice[54]. When FOXO loss is combined with loss of the mTORC1 negative regulator Tsc1 in a mouse model for renal cell carcinoma it was observed that FOXOs do not mitigate the formation of renal adenoma but prevent the progression to renal carcinoma by inducing Mxi1 dependent c-Myc repression [55]. Suppression of the oncogenic effects of c-MYC by FOXOs were also observed in vavP-MYC10;$Foxo3^{-/-}$, Eμ-Myc;$Foxo3^{-/-}$ and Eμ-Myc;$p53^{+/-}$;dnFOXO4 mice. Either loss of Foxo3 or forced expression of dnFOXO4 (a dominant negative version of FOXO4 that comprises only the Forkhead DNA binding domain and has no transcriptional activity but blocks transcription regulation by other Forkhead box proteins) was shown to further promote Myc driven lymphomagenesis [56,57].

Together these studies show that compound loss of Foxo1/Foxo3/Foxo4 alone or in combination with p53 promotes tumorigenesis. FOXOs can additionally fulfill tumor suppressive roles in the context of an oncogenic stimulus like Eμ-Myc, suggesting that FOXOs do not necessarily block tumorigenesis but can obstruct the progression to more aggressive cancer stages.

2.4. FOXOs suppress tumorigenesis

As mentioned above, FOXOs are negatively regulated downstream of the −in cancer − frequently hyper-activated PI3K-PKB/AKT pathway, and activation FOXOs results in cell cycle arrest and apoptosis in various cell lines, FOXOs have been considered to be tumor suppressors [52,58,59]. In line with the observation that FOXOs can repress cell proliferation and induce apoptosis *in vitro*, expression of hyperactive FOXO mutants in xenograft experiments confirmed that primary tumor growth is impaired by ectopic FOXO activation [60,61].

Histopathology studies that focused on correlating FOXO expression and localization to disease outcome in cancer patients emphasize a role of FOXOs as tumor suppressors. Low levels of FOXO1 expression and high levels of the inactivating FOXO1-Thr24 phosphorylation are correlated to reduced overall survival and disease free survival in soft tissue sarcoma, AML, prostate and breast cancer [62–65]. Similarly, high FOXO3 levels correlate with increased disease free survival in clear cell renal carcinoma, colorectal, urothelial, neuroblastoma and breast cancer [66–70]. Pairing primary colorectal cancer samples with their corresponding liver metastases showed that FOXO3 levels in metastasis are significantly lower than in the primary tumor, indicating that lowering FOXO levels may be important for tumor metastasis [66]. FOXO3 levels in ER-positive breast cancer were reported not to influence patient survival and distant metastasis, but in these cases the localization of FOXO3 in the nucleus was correlated to good prognosis and delayed metastasis formation [71]. In line with the observations of FOXO1 and FOXO3, low levels of FOXO4 expression in prostate cancer cells is correlated to poor prognosis and increased metastasis formation [72].

2.5. FOXO mutations in cancer

The fact that at least 4 *FOXO* alleles have to be inactivated to increase tumor incidence renders a frequent loss of FOXO gene function unlikely. While the compound inactivation has indeed never been described, somatic mutations in single FOXO genes have been reported that could contribute cancer in different ways. For example in prostate cancer loss of the genomic region 13q14 containing FOXO1 is commonly observed and FOXO1 was shown to function as a tumor suppressor in this context [73]. Additionally, loss of FOXO3 frequently occurs in Natural Killer cell neoplasms, a rare lymphoid malignancy [74].

Interestingly, the first time a biological function for FOXOs was described, was in the context of proto-oncogenic fusion proteins. Mammalian FOXOs are involved in chromosomal translocations resulting in FOXO1-PAX3/7, FOXO3-MLL or FOXO4-MLL fusion proteins in alveolar rhabdomyosarcoma and acute leukemia [75–77]. In all cases the DNA binding domain of PAX or MLL are fused to the transactivation domain of FOXOs, which result in enhanced expression of PAX and MLL target genes. However, additional oncogenic mutations are required to evoke a cancer-promoting role of these fusion proteins [76,78].

Additionally, single nucleotide somatic mutations in *FOXO1* are enriched in Follicular lymphoma and diffuse large B-cell lymphoma. Interestingly, these mutations are found mostly within the N-terminal region of FOXO1 protein and lead to a shorter form of FOXO1 due to translation initiation from an alternative ATG downstream in the *FOXO1* gene [79]. More detailed examination of FOXO1 mutations revealed that R19, R21 and T24 are frequently mutated [80]. Phosphorylation of FOXO1 by AKT/PKB on T24 is required to induce binding to 14-3-3 proteins and retain FOXO1 in the cytoplasm. The consensus AKT/PKB binding site is RxRxxS/T, indicating that these mutations result in loss of 14-3-3 binding in the case of R19 and R21 mutations and disable phosphorylation of FOXO1-Thr24, all resulting in active FOXO1 [80]. Indeed, the authors described that these mutations increased FOXO1 nuclear localization, suggesting enhanced FOXO1 activity. Contradictory to what one would expect based on FOXOs putative role as tumor suppressors, these mutations were correlated to poor prognosis and point towards a more complex role for FOXOs in cancer.

3. A new paradigm: FOXOs support tumorigenesis

3.1. FOXOs are correlated to bad prognosis

In line with the reported activating mutations in FOXO1 that contribute to tumorigenesis, but in contrast to the histopathology studies discussed above, multiple other reports link high FOXO levels and activation to poor prognosis. In gastric cancer, phosphorylated FOXO1 is correlated to higher overall survival and lower tumor angiogenesis, suggesting that active FOXO1 supports tumor growth and metastasis [81,82].

High expression of FOXO3 is linked to lower overall survival and recurrence free survival in Acute Myeloid Leukemia (AML) patients carrying different types of somatic mutations [83]. Additionally, high FOXO3 expression levels are associated with glioblastoma progression and bad prognosis in pancreatic ductal adenocarcinoma [84,85]. Studies focused on characterizing FOXO3 localization in breast and colorectal cancer described that high levels of FOXO3 nuclear localization correlate to lower overall survival [86,87].

FOXO4 is found upregulated in response to doxorubicin and phenylbutyrate treatment in B-cell lymphoma and FOXO4 expression levels in these cancers are associated with bad prognosis [88].

Finally, high levels of FOXO6 expression was recently correlated to bad prognosis and increased disease progression in gastric cancer [89].

Combined, these data challenge the previously discussed studies that link high FOXO levels and low FOXO activity to good prognosis underlining the controversy on FOXOs function in cancer. Although outnumbered by reports implicating FOXOs to be tumor suppressors, multiple recent studies denote FOXOs as tumor supportive.

3.2. FOXOs partake in metastasis formation

When tumors metastasize cancer becomes lethal. Several studies have connected a supportive role for FOXOs in facilitating and even stimulating metastasis. The first notion of a role for FOXOs in metastasis was in aggressive breast cancer cell lines, which lose their metastatic capacity in xenograft experiments upon knockdown of FOXO3. It was shown that functional loss impairs and activation induces invasive

behavior of breast cancer cells through FOXO3-mediated expression of Matrix Metalloproteinases 9 and 13 (MMP9/13) [90]. Next to FOXO3, FOXO1 has also been linked to regulation of MMP1 expression in breast cancer cells, which could facilitate metastasis [91]. Additionally, in pancreatic ductal adenocarcinoma and glioblastoma xenografts knockdown of FOXO3 restricted both primary tumor growth and metastasis formation [84,85].

FOXOs have also been implicated in colorectal cancer metastasis, as combined nuclear localization of FOXO3 and β-catenin correlates with metastatic disease. FOXO3 hyper activation alone leads to increased cell motility and apoptosis in DLD1 cells, but in the presence of high nuclear β-catenin levels apoptosis is repressed and transplanted cells become metastatic [87]. Conversely, tumors generated from xenografts and patient tumors show efficient drug resistance development against PI3K/PKB/TOR inhibitors in a β-catenin dependent way. Impairing β-catenin accumulation and signaling by co-treatment of these tumors with tankyrase inhibitors significantly reduced this adaptive resistance and increased apoptosis [92]. Together these studies illustrate a more complex role for FOXOs in the formation of metastasis and put FOXOs forward as transcription factors that partake in this process.

3.3. FOXOs in drug response and resistance

Not only do FOXOs fulfill a key role in tumor biology, but are also heavily involved in the response to conventional chemotherapy and small molecule inhibitors. Various studies have reported that FOXOs mediate apoptosis in response to chemotherapeutic drugs including 5-fluorouracil, paclitaxel, resveratrol and inhibitors of BCR-ABL, PI3K or PKB/AKT [93–97]. In contrast, FOXO3 was found to be a key regulator of the Multi Drug Response pump 1 (MDR1/ABCB1), thereby facilitating the acquisition of resistance against Doxorubicin in breast cancer and leukemia cells [98,99].

Next to executing or preventing drug response, the role of FOXOs in maintaining cellular redox homeostasis and elevating oxidative stress resistance contributes to gaining resistance towards drugs that elevate ROS levels in the cells [100]. Also, ovarian cancer cells generate elevated ROS levels as a side effect of paclitaxel treatment and FOXO mediated expression of MnSOD enhances resistance to these elevated ROS levels [101].

Especially in stem cells FOXOs have been described as essential regulators of redox homeostasis and by doing so are required to prevent differentiation and promote survival. Loss of FOXOs in hematopoietic or neuronal stem cells leads to exhaustion of the stem cell pool, reduced self-renewal, a more oxidative redox environment and enhanced proliferation and apoptosis [102–105]. These findings illustrate that FOXOs are essential for tissue homeostasis by balancing redox signaling in stem cells and suggest that FOXOs fulfill a similar role in stem or tumor-initiating cells in cancer.

Inhibitors targeting the common BCR-ABL mutations efficiently reduce tumor load in Chronic myeloid leukemia (CML), but often drug resistant Leukemia Initiating cells (LICs) recur and makes curing the disease problematic. As FOXOs are essential for maintaining hematopoietic stem cells (HSCs), Naka and colleagues assessed whether FOXO3 knockout CML cell populations contain LICs, and if FOXO3 loss limits their capacity to re-establish tumors. Indeed, it was found that LICs lacking FOXO3 have limited tumor reconstitution capacity [106]. The mechanism by which FOXO3 maintains stem cell homeostasis and self-renewing capacity was later ascribed to the regulation of BCL6. As a target gene of FOXO3, BCL6 functions by repressing MYC, p53, Cyclin D2 and CDKN2A and thereby mediates LICs self-renewal capacity [107,108].

A comparable role for FOXOs was found in AML cells carrying an MLL-AF9 fusion protein. LICs within the AML cell population exhibited low PKB/AKT activity and nuclear FOXO localization, implying that FOXOs are active in the LIC population and could play a role in their maintenance. Hyper-activation of PKB/AKT signaling in these AML cells leads to FOXO inactivation and myeloid maturation, thereby reducing the repopulating capacity. Similarly, AML cells knockout for *Foxo1/Foxo3/Foxo4* lose their repopulating capacity in bone marrow transplantation assays and show elevated stress signaling through JNK [109].

Together, these finding show that FOXOs are involved in drug response and are not only required for maintaining healthy stem cell populations but also for tumor-initiating cells in leukemic cancers. Whether FOXOs fulfill a similar role in solid tumors is however still unclear.

3.4. FOXOs mediate growth factor signaling feedback

The use of small molecule inhibitors targeting receptor tyrosine kinases or other oncogenic kinases revealed cancer cells quickly adapt to the loss of oncogenic signaling through elaborate feedback mechanisms that re-establish proliferative signaling. Due to this plasticity, treating patients with inhibitors targeting single oncogenic kinases including PI3K or PKB/AKT is generally ineffective [7,110–112]. The mechanism behind gaining resistance to small molecule inhibitors targeting GF signaling lies in the robust signaling feedback mechanisms triggered upon loss of GF signaling. Signal transduction is not a static process but a balance between the signal and it's feedback. When a GF signaling pathway is activated, the induced negative feedback shuts down the pathway by receptor internalization, inactivation of receptor adapter proteins and reduced expression of RTK signaling pathway components. The other way around, when GF signaling is lost, the cell re-sensitizes itself to GFs by inducing transcription of RTKs, allowing adaptor proteins to be active and localizing RTKs to the cell membrane [113]. As FOXOs are transcription factors regulated downstream of PI3K-PKB/AKT it can be expected that they participate in feedback signaling within this pathway.

Nuclear translocation of FOXOs is a continuous dynamic process as demonstrated by nuclear accumulation of FOXOs in the presence of active growth factor signaling upon blocking nuclear export [6,10,114]. These observations illustrate that FOXOs also do not operate in a binary "ON" and "OFF" fashion but in equilibrium between nuclear import and export. The rate of nuclear import and export is influenced by the multitude of post-translational modifications that can affect FOXOs reported to date (Fig. 1) [3,4]. When growth factor signaling is low FOXO accumulates in the nucleus and induces transcription of genes essential for growth factor signaling cascades including various RTKs, RTK adaptor proteins and regulators of the mTOR complexes, hereby inducing GF signaling feedback.

Mammalian and Drosophila FOXOs can mediate re-establishment of growth factor signaling by regulation of the transcription of the Insulin receptor [115]. Treatment of breast cancer or CML cells with Doxorubicin leads to nuclear localization of FOXO3, which in response upregulates PIK3CA expression and thereby activates PI3K-PKB/AKT. This FOXO3 dependent feed-back signaling to PI3K-PKB/AKT eventually confers doxorubicin resistance to these cells [86,116]. In addition to PI3K, FOXOs regulate both mTORC1 and mTORC2 complexes which inactivate and activate PKB/AKT respectively [117]. Upon activation of FOXO1, either through over-expression or PI3K inhibition, the mTORC2 component RICTOR is transcribed, leading to increased mTORC2 activity and consequent PKB/AKT activity through Ser473 phosphorylation [118]. In parallel, FOXO1 stimulates the expression of SESN3, which in turn inactivates mTORC1 by inhibiting the GTPase RHEB, thereby repressing cell growth but enhancing PKB/AKT activity simultaneously [119].

Furthermore, FOXO3 was found to regulate multiple RTK adaptor proteins including Insulin Receptor Substrate 1/2 (IRS1/2) and Insulin Like Growth factor receptor binding proteins (IGFBP) in human colon carcinoma cells [13]. The role of FOXOs in the regulation of GFR-feedback signaling is not only restricted to the insulin pathway as characterization of RTK expression in response to PI3K-TOR dual

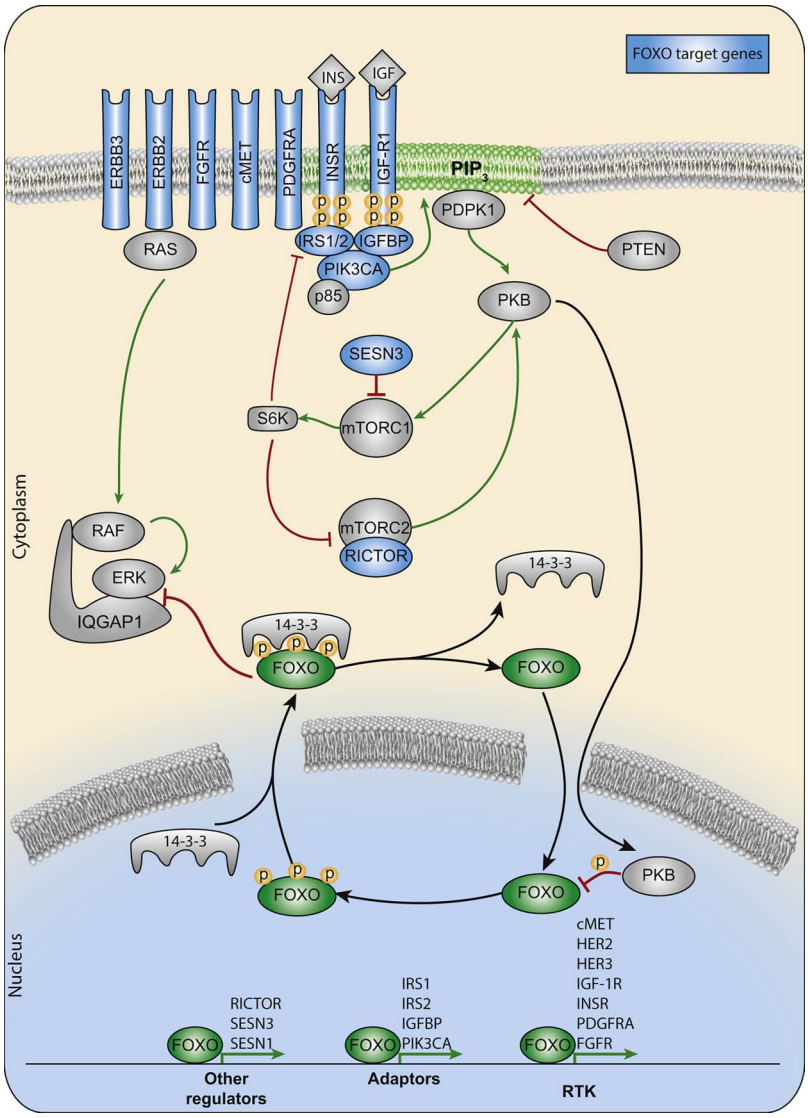

Fig. 2. The growth factor signaling balance between RTK and FOXO activity.
The scheme illustrates the intrinsic growth factor signaling feedback loop that balances RTK activity with FOXO activity. Proteins in blue are up-regulated in response to FOXO activation.

inhibitors revealed that FOXOs can drive the expression of different RTKs including MET, ERBB2, ERBB3 and RET in breast cancer and are part of the mechanism behind development of resistance to PI3K-PKB/AKT-inhibiting drugs [120].

In addition to PI3K feedback signaling, FOXOs were recently described to activate the RAS-MEK-ERK pathway through binding to IQGAP1, a scaffold protein involved in activation of this pathway. Cytoplasmic FOXOs bind to IQGAP1 and thereby prevent the activation of ERK. Upon translocation to the nucleus in response to loss of PI3K-PKB/AKT signaling, the binding of FOXOs to IQGAP1 is lost allowing ERK to become active. This results in compensation for the loss of GFR signaling and yields resistance to PI3K inhibitors and paclitaxel [121].

It is clear that the mechanism by which the cell mediates activity of FOXOs requires balancing of nucleo-cytoplasmic shuttling (Fig. 2). However, experiments scrutinizing the exact endogenous dynamics of FOXO shuttling are lacking to date. Understanding the dynamics of endogenous FOXO shuttling and intervening with these dynamics might hold interesting insights especially for cancer therapy.

4. Revising the role of FOXOs in tumorigenesis

4.1. Context dependent transcriptional output by FOXOs

The observations summarized above implicate that, dependent on the context; FOXOs can either act as tumor supporters or suppressors. For instance, even a mild induction of CDKis or pro-apoptotic factors by FOXOs may be enough to tip the proliferation/apoptosis balance towards net cell death. But once a tumor reaches a stage at which it is insensitive to these aspects of FOXO function, it may actually benefit from the role of FOXOs in preventing the accumulation of high ROS levels and maintaining bioenergetics. The observation that aggressive cancer types often rely on activated growth factor signaling and consequentially repress FOXO activity also suggests that tumors generally restrict FOXO activity to a certain extent.

Especially in metastasis formation fine-tuning of the activity and output of FOXOs seems crucial an is influenced by the spatial localization of cells, concurrent signaling and the epigenetic landscape. For example, a dichotomous response was observed in breast and ovarian cancer spheroids in which PI3K was inhibited, showing apoptosis only in cells on the inside of the spheroids. Within these spheroids a clear FOXO related gene expression profile was observed, but dependent on the location of the cell in the spheroid at the moment of PI3K inhibition the effect of induced transcription was different [122]. In line with this environment-dependent dichotomy, it was found that breast cancer cells require PKB/AKT-mediated inhibition of FOXOs in order to prevent BMF-dependent anoikis. Interestingly, FOXO3 binding to the *Bmf* locus was enhanced in cells cultured in suspension compared to cells cultured under adherent conditions, suggesting that the *Bmf* locus

becomes more permissive for FOXO binding depending on cellular context. Therefore, FOXO activity in anchorage-independent conditions might be sufficient to induce anoikis while under adherent conditions FOXO activity is insufficient with respect to apoptosis induction [46].

Low levels of FOXO activity might be expected in detached cells even in the presence of GFR signaling. It is well described that anchorage-independent survival is stressful for cells as glucose uptake is impaired and high levels of ROS are present, two situations in which FOXOs can become activated. As cells cultured in suspension require active GFR signaling to survive, it might also be plausible that active ERK and PKB/AKT stimulate survival by inhibiting pro-apoptotic protein activity through phosphorylation, allowing FOXOs to be active and contribute to ROS detoxification and maintaining bioenergetics, as pro-apoptotic genes remain post-translationally inactivated [47,123].

Next to cellular context, it was shown that high levels of β-catenin protein expression and co-localization with FOXO3 suppresses the induction of pro-apoptotic genes and stimulates the expression of genes involved in metastasis [87] Another example of co-factor-dependent tumor supportive behavior of FOXOs was found in MCF7 breast cancer cells that are either positive (ER+) or negative (ER-) for the estrogen receptor. FOXO3 hyper-activation in ER+ cells results in suppression of cell proliferation, migration, invasion and anchorage independent growth. These effects are reversed in MCF7 cells treated with RNAi targeting ER, resulting in ER- MCF7 cells [124].

Combined these studies establish a complex role for FOXOs in tumor development and progression, and demonstrate that a dual tumor enhancer and suppressor role for FOXO is not necessarily contradicting, but that the context in which FOXOs are activated dictates the observed phenotypes (Fig. 3).

4.2. FOXOs are not tumor suppressors

FOXOs are tumor suppressors: this is the textbook view attributed to FOXOs in the context of their role in cancer. The adjective *bona fide* is commonly used for tumor suppressor genes in literature and implies these genes faithfully repress tumorigenesis, suggesting that genes like FOXO exist to prevent cancer. However, whether the biological role of tumor suppressor proteins is truly focused on repressing cancer or that the tumor suppressive effects are just a side effect of their normal function is debatable, certainly for FOXOs.

In parallel to the findings that put FOXOs forward as tumor suppressors, various studies have now been published on the homeostatic functions of FOXOs in both healthy and tumor tissues, as discussed in this review [23,100,102,105](reviewed in: [3,4,11,16]). There is no reason to assume that any molecule (including FOXO) discriminates between tumor cells and healthy cells in fulfilling homeostatic functions [106,109]. Additionally, FOXOs were found to facilitate breast cancer cell motility, stimulate metastasis of colorectal cancer cells and to participate in development of resistance to PI3K and PKB/AKT inhibitors in various cell lines [87,90,118–120].

In contrast to classical tumor suppressors like PTEN, RB or E-cadherin, FOXOs are rarely lost or mutated in a way that activity is fully compromised, one might therefore hypothesize that tumors could benefit from retaining FOXO function. It even becomes tempting to speculate that endogenous levels of FOXO activity are not tumor suppressive when it comes to repressing cell proliferation, inducing apoptosis and metastasis. Whether endogenous FOXOs can confer a tumor suppressive phenotype has not been studied outside the context of PKB/AKT inhibition or overexpression of FOXOs: situations in which many other protein functions change or FOXO activity is unnaturally high respectively. Based on the observation summarized above, the suggestion that FOXOs are classic *bona fide* tumor suppressors clearly needs revision. Rather, FOXOs function as factors that support cellular resilience in both healthy and cancer cells.

4.3. Using FOXO in the clinic

Although FOXOs have been implicated in major diseases like cancer and diabetes, developing targeted therapies to activate or inactivate FOXOs or using FOXOs as diagnostic markers has been proven difficult. Histopathological studies that aimed at correlating FOXO expression and localization to disease outcome in cancer patients have yielded contradicting results. On one hand many studies observed a correlation between high FOXO expression and/or nuclear localization and good prognosis [62–69,71,72]. Conversely, various studies have implicated that high FOXO levels and/or activity correlate to poor prognosis [81–83,86,87,125]. Although these studies come to contradictory conclusions, we noticed that in retrospect more technical and biological IHC controls should have been incorporated to substantiate these observations. In addition, many papers do not report the antibodies used, making interpretation and reproduction of these studies difficult. It is therefore advisable to be cautious drawing conclusions based on the current knowledge from histopathological studies on FOXO levels and localization.

Assessment of FOXO activity prior and after patient treatment could be interesting in light of increasing therapy efficiency as FOXOs are involved in the acquisition of therapy resistance. Histopathological examination of FOXO localization or phosphorylation by PKB/AKT alone in this perspective is not expected to be a suitable approach as we discussed that even when FOXOs are expected to be inactive and cytoplasmic in tumors based on the tumors dependency on active growth factor signaling, this is not necessarily the case for 100% of FOXO molecules. Considering the above, determination of the expression levels of FOXO target genes combined with FOXO localization in tumor samples is likely more informative to determine the actual status of FOXO activity [126,127]. As FOXO localization is roughly inversely correlated to PI3K activity, the localization of FOXO has also been suggested as a measurement of PI3K pathway activity status in the tumor [128].

The role of FOXOs in cancer cell etiology and their pivotal role in regulating growth factor signaling feedback makes activation of FOXOs as a putative strategy for cancer therapy questionable and suggests that inhibition of FOXOs might be a more sensible strategy. Combining FOXO inhibitors with drugs targeting PI3K might then aid in preventing resistance. The expected FOXO induced cell death and cell cycle arrest downstream of PI3K-PKB/AKT inhibition might in this case be reduced. However, the PI3K pathway functions as a strong survival and proliferation signal by directly regulating the activity multiple apoptotic and cell cycle factors independently of FOXOs and might therefore still be an effective target even when FOXOs are inhibited [123,129].

Attempts to design FOXO-specific inhibitors are limited but have been reported. AS1842856 is the only commercially available small molecule inhibitor allegedly targeting FOXOs and should prevent translocation to the nucleus [130]. AS1842856 has mainly been tested in the context of diabetes, bone development, adipocyte differentiation and pulmonary hypertension [131–134]. In cancer, AS1842856 was found to prevent the induction of senescence in progesterone receptor positive ovarian cancer and block MYC-driven lapatinib resistance in breast cancer [135,136]. It remains to be characterized however, whether FOXO translocation is fully inhibited by AS1842856 in the absence of GF signaling or in an oxidizing redox environment. Further characterization of the compound and the development of additional pan-FOXO inhibitors seems nonetheless worthwhile based on the promising effects of inhibition of FOXOs in cancer (Fig. 3).

Next to small molecule inhibitors two peptides have been reported to attenuate FOXO function. A small FOXO1 mimicking peptide was developed that can bind IQGAP1 in a similar fashion as endogenous FOXOs, preventing ERK binding and activation. As a result, feedback activation of ERK upon FOXO translocation is inhibited and the acquisition of drug resistance against PI3K and paclitaxel is blocked in prostate cancer cells [121]. Another small peptide mimicking FOXO4

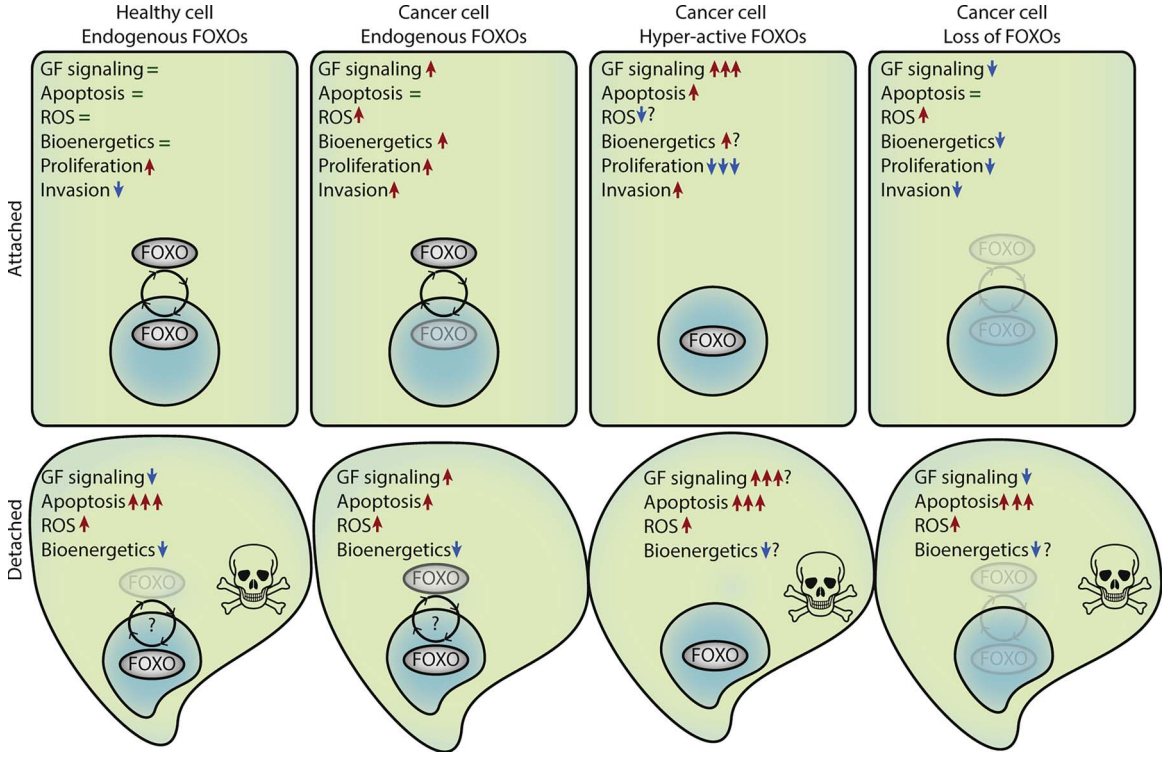

Fig. 3. Context dependent FOXO output.
Illustrated are the reported effects of different levels of FOXOs activity on growth factor signaling, apoptosis, ROS levels, bio-energetics, proliferation and invasion in the primary tumor or healthy tissue (attached) and cells that are disseminating or accidentally detached (detached). Arrows indicate if processes are activated or inhibited. Question marks (?) indicate processes that are not well understood to date.

was developed recently. The peptide targets the FOXO4-p53 interaction interface, hereby blocking FOXO4 binding to p53. Under normal conditions, FOXO4 and p53 binding is required for cells to maintain a senescent state after severe DNA damage. Blocking p53 binding to FOXO4 in senescent cells results in the induction of apoptosis and clearance of senescent cells from the body. Whether this peptide can also affect cancer cells has not been reported yet but might hold interesting insight in the role of FOXOs in cancer [137].

5. Conclusion

Taken together, a picture emerges in which the role of FOXOs in cancer is more supportive than commonly thought. The studies showing a tumor supportive role for FOXOs allow a new perspective that can be used for novel insights into tumorigenesis and the involvement of the PI3K-PKB/AKT pathway in this process. The role of FOXOs in cancer is complex however and further understanding of how endogenous FOXOs are contributing to cancer progression is required. Studies are needed in which basic questions concerning tumor enhancing or suppressive functions can be addressed using robust and clinically relevant cancer models. Additionally, some studies have already reported beneficial effects of repressing FOXOs in cancers, but pharmacological tools to effectively interfere with FOXOs are scarce and still under development. Fundamental understanding of how FOXOs are regulated and how they generate specific transcriptional output will allow the design of drugs targeting FOXOs and open up new ways for future combinational therapies with existing PI3K/AKT antagonists inhibitors in the future.

Conflict of interest statement

The authors declare that there are no conflicts of interest

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