APPLICATION NOTE

qPCR gene expression: Detection of related AKT protein kinase family members in an RNAi-based study of FOXO1 regulation.

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Abstract

Quantitative Real-time PCR (qPCR) is a routine laboratory technique for the measurement of DNA or RNA that is both sensitive and specific with appropriate assay design. Solaris qPCR Gene Expression Assays are ideal for routine molecular applications because they combine the Epoch™ Minor Groove Binder™ Technology (MGB; Epoch Biosciences Inc.) with a rigorous algorithm that permits detection of all known splice variants of a gene target, while distinguishing among closely related family members, on a genome-wide scale. Here we describe the application of this new probe/primer qPCR detection technology in a study that deciphers the individual contributions of the highly related gene family members, AKT1, AKT2 and AKT3, in regulating the activation and redistribution of the Forkhead/FOXO1 transcription factor.

Introduction

Protein kinase B (PKB) otherwise known as AKT is a serine/threonine protein kinase that acts as a central node in multiple cell signaling pathways1. The AKT family of proteins is comprised of three highly homologous members sharing structural and sequence conservation: PKBα/AKT1, PKBβ/AKT2 and PKBγ/AKT3. The family members are induced following activation of the phosphatidylinositol-3 kinase (PI3K) signaling pathway with growth factors, cytokines and other cellular stimuli. Phosphorylation of AKT members triggers their activity where they in turn phosphorylate a spectrum of substrates within the cell. One class of these substrates includes the FOXO transcription factors, which are members of the Forkhead family (FKHR) of transcription factors characterized by a conserved DNA-binding domain[2].

FOXO transcription factors, comprised of four members in mammals (FOXO1, 3, 4 and 6), are at the nexus of critical cellular processes such as apoptosis, cell-cycle progression, oxidative stress, glucose metabolism and energy homeostasis[3]. Phosphorylation of FOXO by activated AKT leads to the retention of FOXO within the cytoplasm and therefore results in its inactivation (Figure 1A). Inhibition of the AKT activity within the cell (such as withdrawal of survival factors) leads to FOXO dephosphorylation, nuclear translocation and activation of its target genes (Figure 1B)[4].

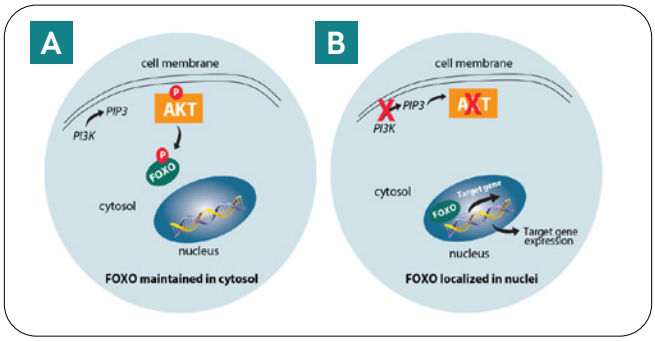

Figure 1: The AKT signaling pathway. Growth factor receptors recruit phosphatidylinositol-3 kinase (PI3K) to the cell membrane and induce the production of second messengers phospatidylinositol-3,4,5-triphospahtate (PIP3) that convey signals from the cell surface to the cytoplasm. AKT binds these second messengers at the plasma membrane where it gets phosphorylated and activated by the kinase 3-phosphoinositide-dependent protein kinase. Proteins phosphorylated by activated AKT regulate diverse cellular functions. These include certain members of the FOXO family of transcription factors resulting in their retention in the cytosol **(A).** Inactivation (X) of the PI3K/AKT pathway results in FOXO dephosphorylation, nuclear translocation and transcriptional activation of its target genes **(B).**

The AKT protein family members are broadly expressed in most organs and tissues; AKT1 is ubiquitously expressed at high levels; AKT2 is highly expressed in insulin-sensitive tissues such as the liver, skeletal muscle and adipose tissue and AKT3 is the predominant form expressed in the brain and testis[5]. AKT kinases are involved in regulating critical cellular processes such as apoptosis, cell growth, differentiation and energy metabolism. Having such a diverse role in many normal processes, it is not surprising that AKT dysfunction is intimately involved in numerous disease states including diabetes and cancer[6,7]. Specifically, it has been shown that isoform-specific functions of AKT family members can contribute to tumorigenesis on multiple levels; this is perhaps expected given the original discovery of AKT as the protein encoded by the cellular homolog of the viral oncogene v-akt[8]. Numerous studies have since examined oncogenic amplifications of different AKT genes in primary human tumors and cancer cells.

For example, AKT1 gene amplification and mutation occurs in gastric and colorectal cancer and amplifications of AKT2 are known to affect breast, ovarian and pancreatic cancer[9].

Conservation of the AKT family members suggests potential for redundant functions of the AKT family members. For this reason, to ascertain their individual and combinatorial biological effects, it is crucial to have molecular tools capable of selectively modulating and detecting their specific roles. Such tools can enhance our understanding of the relative contribution of the AKT isoforms to different biological processes and might open a door to developing selective modes of therapy.

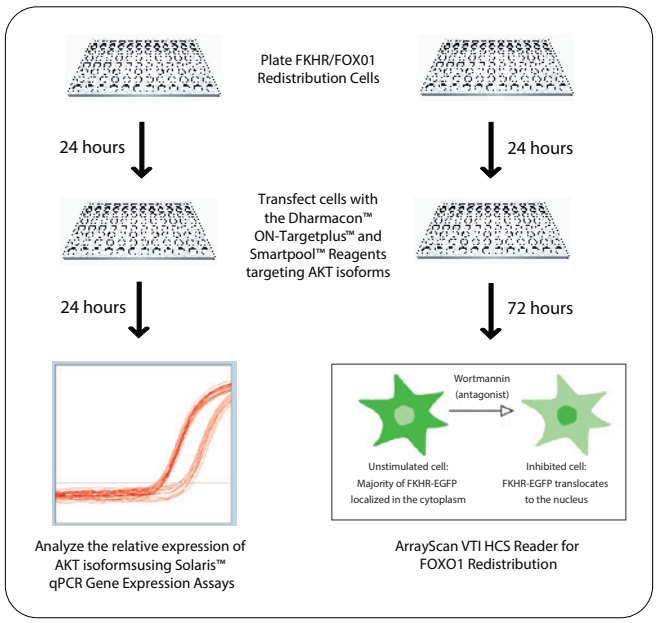

Figure 2: Experimental workflow. Dharmacon™ ON-TARGET*plus*™ SMARTpool™ siRNAs targeting either AKT1, AKT2 or AKT3 or a combination of them were transfected into the FKHR redistribution cell line, a recombinant U2OS cell line that stably expresses human FKHR/FOXO1 fused to the N-terminus of enhanced green fluorescent protein (EGFP). Duplicate micro-well plates were transfected to determine the relative AKT expression at 24 hours post-transfection by Solaris qPCR Gene Expression Assays, and for high-content analysis for the FOXO1 redistribution at 72 hours post-transfection on the Cellomics ArrayScan VTI HCS Reader by monitoring the translocation of a FKHR-EGFP fusion protein from the cytoplasm to the nucleus.

Here we describe the use of RNA interference (RNAi) technology to down-regulate the expression of each specific human AKT family member to examine their isoform-specific roles on the regulation of FOXO1 in a Thermo Scientific FKHR Redistribution© cell line, engineered to assess FOXO1 protein translocation by high content analysis. Specificity of the down-regulation of each family member was assessed using Solaris qPCR Gene Expression Assays.

These probe-based qPCR assays represent probe/primer sets that are generated using a novel, tier-based algorithm to create reagents that detect all known splice variants of a given gene yet are capable of distinguishing even closely related family members. The Solaris qPCR Gene Expression Assays incorporate Epoch Biosciences MGB[10], and Superbase™ (Epoch Biosciences Inc.) technologies for increased sequence design space and enhanced specificity. MGB probes hybridize more strongly to their complementary sequences than standard DNA probes and display an increased melting temperature allowing for the use of shorter but highly specific probes. In addition, the modified nucleic acid bases (Superbases) can be substituted in primer and probe design to raise the melting temperature and eliminate many of the problems associated with AT- or GC-rich regions. Incorporation of these two chemical strategies with a fluorescent (FAM) reporter dye and corresponding Eclipse™ Dark Quencher™ fluorochrome results in high performance assays that consistently function under universal thermal cycling conditions.

Results

To study the roles of the closely related AKT family members on FOXO1 regulation, we used an RNAi approach to down-regulate the AKT family members in a U2OS FKHR Redistribution cell line (Figure 2). These recombinant cells stably express human FKHR/FOXO1 fused to the N-terminus of the enhanced green fluorescent protein (EGFP) for easy monitoring of the FOXO1 relocation. Using high content analysis (Thermo Scientific™ Cellomics™), one can track the consequences of inhibiting the AKT pathway. Cells plated in 96-well micro-titer plates were transfected in triplicate with ON-TARGET*plus* siRNA and SMARTpool reagents against individual or combinations of the AKT family members, including down-regulation of all three kinases simultaneously. Replicate micro-titer plates were prepared to determine gene target expression levels as well as high-content analysis. This approach allowed for quantification of FOXO1 Redistribution from the cytoplasm to the nucleus on the Thermo Scientific™ Cellomics ArrayScan™ VTI HCS Reader as well as correlative detection of AKT gene expression.

Alternatively spliced variants have been described for the AKT family of kinases: AKT1 has three splice variants that differ in the 5′-untranslated region (UTR) but encode the same protein and AKT3 has two splice variants that differ in the 3′-end region, which includes a part of the coding sequence, resulting in two protein variants with a different C-terminus. AKT1, AKT2 and AKT3 mRNAs share a high degree of homology: 80% sequence identity between AKT1 and AKT2, 70% sequence identity between AKT3 and AKT2 and 71% sequence identity between the AKT1 and AKT3 mRNA coding regions. Figure 3A depicts a schematic representation of the AKT1, AKT2 and AKT3 transcripts with all splice variants and the positions and size of the amplicons obtained using the Solaris qPCR Assays. Figure 3B shows the complementarities of the AKT2 Solaris Assay with the sequences of all three AKT transcripts. Both primers and the probe have perfect complementarities only to the AKT2 but not the AKT1 or AKT3 sequences. Since Solaris Assays are designed using a rigorous algorithm to specifically detect all known splice variants of a gene target, they represent an ideal tool to measure RNAi-mediated changes in the expression of each AKT family member. The design algorithm also incorporates preferences for assays to span exon-exon junctions where the primer, probe or amplicon crosses an exon-exon junction. Given the fact that all three kinases are highly homologous, primers and probe of the qPCR assay must be designed in regions where the sequence differences would allow for specific amplification of only that target which has perfect complementarities to the primer/probe set. The available isoform-specific sequence that can be utilized for assay design may become limiting when designing assays to gene targets with multiple splice variants and family members. This restriction can be resolved by adjusting the annealing temperature through the incorporation of the MGB moiety and Superbases. This design strategy effectively increases the sequence design space and permits design of gene-specific Solaris Assays.

We used the AKT1, AKT2 and AKT3 Solaris qPCR Assays to measure the siRNA-mediated changes in expression of the endogenous levels for each AKT family member. Figure 4 illustrates that specific siRNAs designed against each of the AKT family members results in isoform-specific mRNA knockdown (red bars) compared to associated control groups (blue bars). A SMARTpool siRNA reagent targeting AKT1 led to down-regulation of the target mRNA by > 90% with no effect on the mRNA levels of AKT2 or AKT3 (Figure 4A). Similarly, the AKT2 SMARTpool siRNA regeant resulted in a knockdown of AKT2 mRNA by ~ 95% with no effect on the other family members (Figure 4B) while the siRNA pool against AKT3 led to a knockdown of the AKT3 mRNA by 90% with no effect on AKT1 or AKT2 levels (Figure 4C).

Furthermore this specific knockdown was maintained when combinations of siRNA pools against two of the three kinases was used for a double knockdown or all three for a triple knockdown of AKT family members. For example, when siRNA pools against AKT1 and AKT2 were combined, there was specific target mRNA knockdown of AKT1 and AKT2 but not AKT3.

Combinations of the siRNA pools targeted against all three AKT family members resulted in an efficient knockdown of all three family members. Figure 4D shows the log scale amplification curves obtained with Solaris qPCR Assays for AKT1, AKT2, AKT3 and a reference gene, GAPDH. Samples transfected with siRNA against the particular AKT isoform assayed by the Solaris Assay show a shift in the amplification curves by greater than three Cq values. In contrast, the Cq values for GAPDH remained the same across all samples, indicating no change in the relative expression across distinct transfected samples. These values were used to normalize the expression of the AKT isoforms across the silencing experiments. The expression analysis clearly shows that mRNA knockdown was achieved by the isoform-specific siRNAs and could be detected by the corresponding isoform-specific Solaris Assays.

Now with the appropriate tools in place to discern the gene specific functions, the next step is assay selection. We chose the FKHR Redistribution Assay, which is ideally suited to screen for modulators of the FOXO1 transcription factor by monitoring the cellular redistribution of the FOXO1-EGFP fusion protein (Figure 5). In dividing cells, FOXO1 is phosphorylated by the AKT kinases and is sequestered in the cytoplasm in its inactive form, as illustrated in Figure 5A. Upon inhibition of the AKT pathway the FOXO1 phosphorylation and cytoplasmic sequestration is hindered resulting in nuclear accumulation. Wortmannin, a common PI3K covalent inhibitor, inhibits the PI3K-AKT signaling pathway and results in a nuclear distribution of FOXO1 (Figure 5B). We examined the effects of down-regulation of individual or combinations of the AKT family members on the nuclear redistribution of FOXO1.

In cells transfected with a non-targeting siRNA control (NTC) the FOXO1 is localized primarily in the cytoplasm similar to the untreated cells (Figure 5C). Although robust silencing of the individual AKT family members was achieved, there were no significant changes in the redistribution of FOXO1 when each of the AKT kinases was individually own-regulated (Figure 5E). This supports previous observations that there is a level of redundancy inherent within the PI3 kinase/AKT signaling system[5].

When AKT2 and AKT3 family members were down-regulated together, there was an approximately six-fold induction of FOXO1 redistribution to the nucleus (Figure 5E). However, more than a ten-fold increase in redistribution of FOXO1 from the cytoplasm to the nucleus is observed when siRNA pools were combined to simultaneously knockdown all three AKT family members (Figure 5D and E). This is about 25% of the response observed with the positive compound control wortmannin, which likely reflects the fact that reference compounds act differently than siRNAs at a biological level. For example, timing, potency and effects on the total enzyme pool are distinct for a small molecule compound as compared to siRNAs. Further, wortmannin has been shown to have a high degree of toxicity and non-specific effects on other kinase signaling pathways that may enhance the apparent phenotypic effect. Our data clearly indicate that FOXO1 translocation in the U2OS cell line is regulated by all three AKT family members, with AKT2 and AKT3 having some predominance in the regulation.

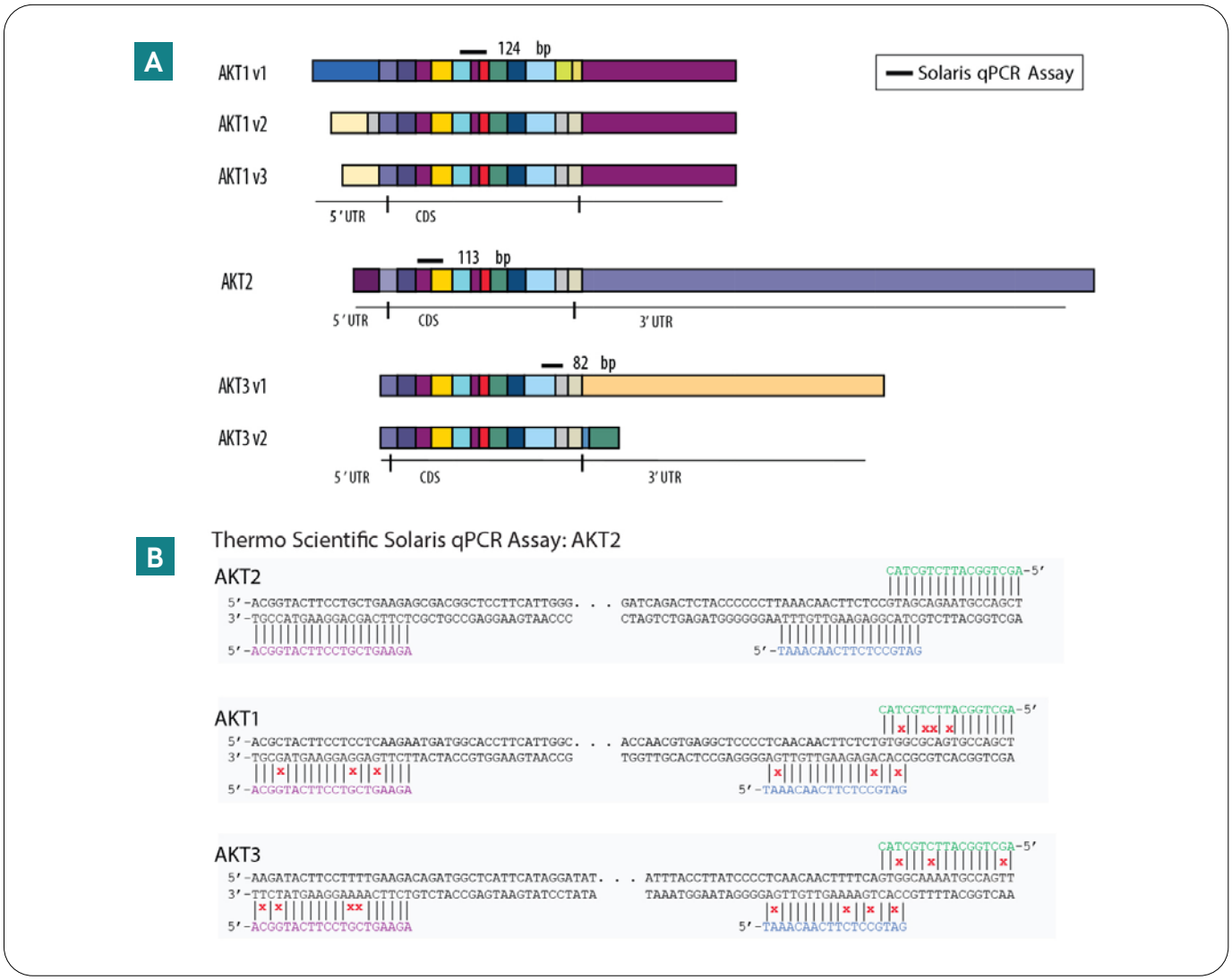

Figure 3: Schematic representation of the AKT1, AKT2 and AKT3 transcript variants and Solaris qPCR Assay positions with sequence alignment. **(A)** AKT1 has three splice variants (v1, v2, v3), AKT2 has one mRNA and AKT3 has two splice variants (v1, v2). The different color bars correspond to different exons for each AKT gene. The amplicon positions and size produced by the Solaris qPCR Assays are represented by the black bars. All three Solaris Assays are exon spanning. **(B)** Sequence complementarities of the AKT2 Solaris Gene Expression Assay to the AKT1, AKT2 and AKT3 transcripts (primers shown in purple and green, probe shown in blue).

Discussion

The three members of the AKT family of protein kinases have been implicated in a plethora of cellular signaling processes with key functions in the control of cellular metabolism, growth, proliferation and apoptosis. Defects in the AKT signaling underlie various human diseases including cancer and diabetes. Thorough insight into the isoform-specific roles of AKT family is essential to fully understand the degree of functional redundancy between the family members and their relative contributions to diverse biological processes and diseases. This could ultimately lead to the derivation of isoform-specific targeted therapies. Subsequent development of isoform-specific mouse knockout models and more recently, the use of isoform-specific siRNA, have improved our understanding of AKT regulation and the roles of the different isoforms in distinct cellular processes [reviewed in[6]]. For example, disruption of the *Akt* genes in the mouse germ line results in isoform-deficient mice displaying very specific phenotypes. *Akt1* null mice show placental hypotrophy, with accompanied retardation of growth and reduction of body weight when compared to wild-type littermates. *Akt2* null mice become hyperinsulinemic and hyperglycemic and *Akt3* null mice exhibit reduced brain sizes. The fact that all three knockout mice strains are viable and demonstrate only subtle phenotypes suggests that all three isoforms compensate for each other.

In support of this *Akt1/Akt2* null mice show severe dwarfism, atrophy of multiple organ systems that include the skin and skeletal muscle resulting in an early neonatal lethality. *Akt2/Akt3* double null mice, although viable, exhibit impaired glucose homeostasis and growth deficiencies5. Support for the existence of both redundant and distinct functions of AKT isoforms has also emerged from the analysis of isoform-specific siRNA-mediated AKT knock-downs in adipocytes. While knockdown of either AKT1 or AKT2 led to a comparable defect in insulin-stimulated glycogen synthesis, similar to the phenotype displayed in knockout mice, AKT2 knockdown had a more prominent effect on insulin-stimulated glucose uptake[11].

Herein we used the AKT family as an example of investigating closely related members with both redundant and distinct functions by coupling RNAi tools to specifically modulate gene function with a novel detection technology to measure the consequences of isoform-specific modulation. AKT family members share extensive sequence similarity: 80% sequence identity across the coding region between AKT1 and AKT2 and 70% or 71% sequence identity between AKT3 and AKT2, or AKT1, respectively. This represents a formidable challenge for the design of the appropriate siRNA and qPCR reagents. The ON-TARGET*plus* siRNAs pools were used to down-regulate the expression of the individual AKT family members and the consequences measured by monitoring the localization of FOXO1, a major downstream substrate. A novel primer/probe-based qPCR methodology, Solaris qPCR Gene Expression Assays, was used to assess the down-regulation of the expression of the AKT isoforms. We found that AKT family members indeed exhibit redundant function in the regulation of the downstream substrate FOXO1 and that down-regulation of all three isoforms is necessary to inhibit the AKT signaling to FOXO1. Now armed with these powerful molecular tools, researchers can extend their investigations to different biological systems to help decipher the role of highly related family members on the regulation of different downstream targets and better understand gene specific regulation and roles in normal and diseased human states.

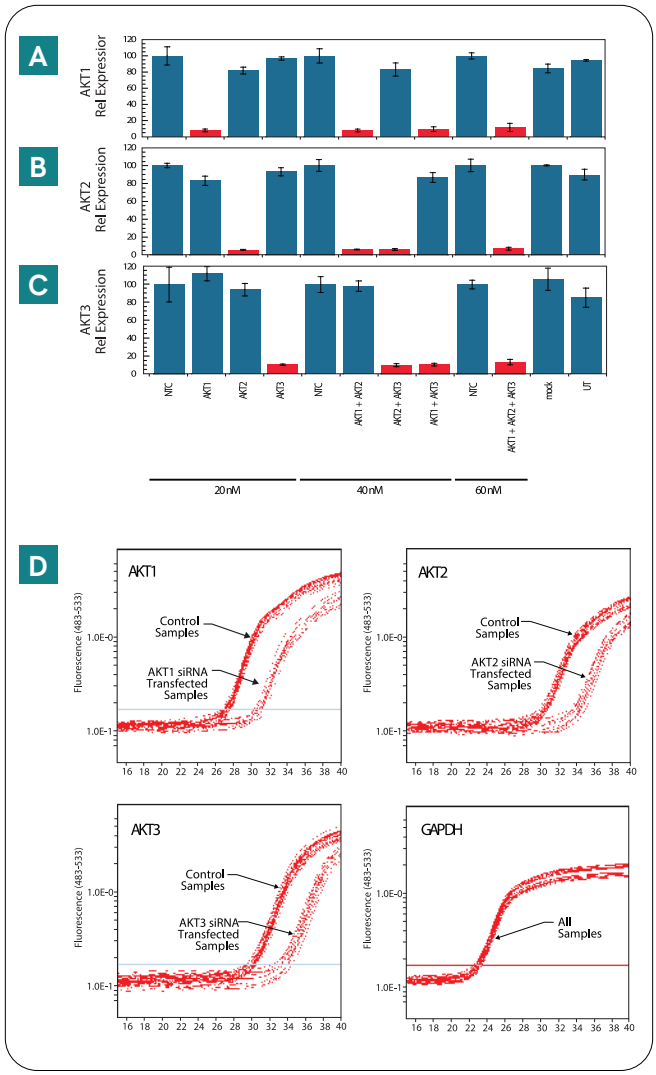

Figure 4: Analysis of the AKT knockdown by siRNA using Solaris qPCR Gene Expression Assays. **(A–C)** Relative expression of AKT isoforms following siRNA transfection: qPCR was performed using Solaris qPCR Assays for detection of AKT1, AKT2, AKT3 and GAPDH on a Roche™ LightCycler™ 480 (384-well) instrument. Knockdown of AKT1 **(A)**, AKT2 **(B)**, and AKT3 **(C)** was calculated using a ΔΔCq method [normalized to GAPDH reference gene and Non-targeting Control (NTC) treated cells at the corresponding siRNA concentration]. Treatment of the cells with siRNAs against each of the AKT isoforms results in the isoform-specific mRNA knockdown (red bars) compared to associated control groups (blue bars). **(D)** Log scale amplification curves obtained with Solaris qPCR Assays for AKT1, AKT2, AKT3 and GAPDH: samples transfected with siRNAs against the particular AKT isoform being assayed by the Solaris qPCR Assay show a shift in the amplification curves by > 3 Cq values. GAPDH was used as a reference and shows no difference in C_q values among all samples.

Methods

RNA interference (RNAi)

The FKHR Redistribution cell line (Thermo Scientific Cat #008_01) is a recombinant U2OS cell line that stably expresses human FKHR/FOXO1 fused to the N-terminus of enhanced green fluorescent protein (EGFP). Cells were cultured under recommended medium conditions and plated in 96-well micro-well plates 24 hours prior to transfection at a density of 2,000 or 10,000 cells per well for high content imaging and for knockdown analysis, respectively. Cells were transfected with the ON-TARGET*plus* SMARTpool reagents (20-60 nM final concentration) using the DharmaFECT™ 3 transfection reagent (Cat #T-2003-03) at the optimized final concentration for each cell density: (0.075 µL/well or 0.3 µL/well). Parallel plates were transfected and analyzed by qPCR at 24 hours post-transfection or by high-content microscopy at 72 hours post transfection. The siRNAs used for transfection included: ON-TARGET*plus* SMARTpool against AKT1 (Gene ID: 207), AKT2 (Gene ID: 208) and AKT3 (Gene ID: 10000) (Dharmacon Cat #L-003000-00; L-003001-00 and L-003002-00, respectively). When combinations of ON-TARGET*plus* SMARTpool reagents were used against AKT family members the final siRNA pool concentration against each AKT member was maintained at 20 nM. Untreated, lipid only and cells transfected with the ON-TARGET*plus* Non-Targeting siRNA Pool (Dharmacon Cat #D-001810-10) (at 20 nM, 40 nM and 60 nM final concentrations) were used as negative controls.

Quantitative real-time PCR (qPCR)

RNA was isolated using Promega SV 96 Total RNA Isolation System (Cat # Z3505). cDNA synthesis was performed using Thermo Scientific™ Verso™ cDNA synthesis kit (Cat # AB-1453/B). Gene expression analysis was performed using Solaris qPCR Gene Expression Assays [(Cat #AX-003000-00-0100 (AKT1), AX-003001-00-0100 (AKT2), AX-003002-00-0100 (AKT3) and AX-004253-00- 0100 (GAPDH)] and Master Mix (Cat #AB-4350/C), which includes an inert blue dye for visualization. qPCRs were carried out in 12.5 µL reaction volume with final oligonucleotide concentrations of 800 nM each primer and 200 nM of MGB-probe. A standard qPCR thermal cycling protocol was employed (DNA polymerase activation at 95 °C, 15 minutes, 1 cycle; denaturation at 95 °C, 15 seconds, annealing/extension at 60 °C, 60 seconds, 40 cycles). Samples were run on a LightCycler™ 480 in 384-well white plates (Roche Cat #04729749001). Expression data was normalized to a GAPDH reference gene using a ΔΔCq method. Expression levels were further normalized to Non-targeting Control siRNAs and are reported as a percentage of the Non-targeting Control expression level.

High-content analysis

TFKHR-U2OS cells transfected as described above and incubated for 72 hours were fixed with 4% paraformaldehyde and stained with Hoechst 33258 (Molecular Probes, Cat #H1398) for subsequent high-content analysis. Un-transfected wells were treated with 150 nM wortmannin (Calbiochem, Cat #681675) for 1 hour prior to fixation, used here as a positive compound control that is known to induce robust nuclear redistribution of FOXO1[12]. The 96-well micro-well plates were imaged using a 10x objective (0.63X coupler), XF100 filter sets for Hoechst and FITC and the Thermo Scientific™ Redistribution™ V3 BioApplication software. Three replicate wells for each siRNA transfection or treatment were analyzed. The data output used was the mean log of the ratio of average fluorescence intensities of the nucleus and cytoplasm. Five fields in each well were measured (~ 300 cells/field). The averages and standard deviations were calculated for each treatment and numeric data evaluated with the vHCS™ Discovery Toolbox. The data was further normalized and presented as percent of the effect of 150 µM wortmannin. For cellular images, plates were re-scanned using a 20× objective.

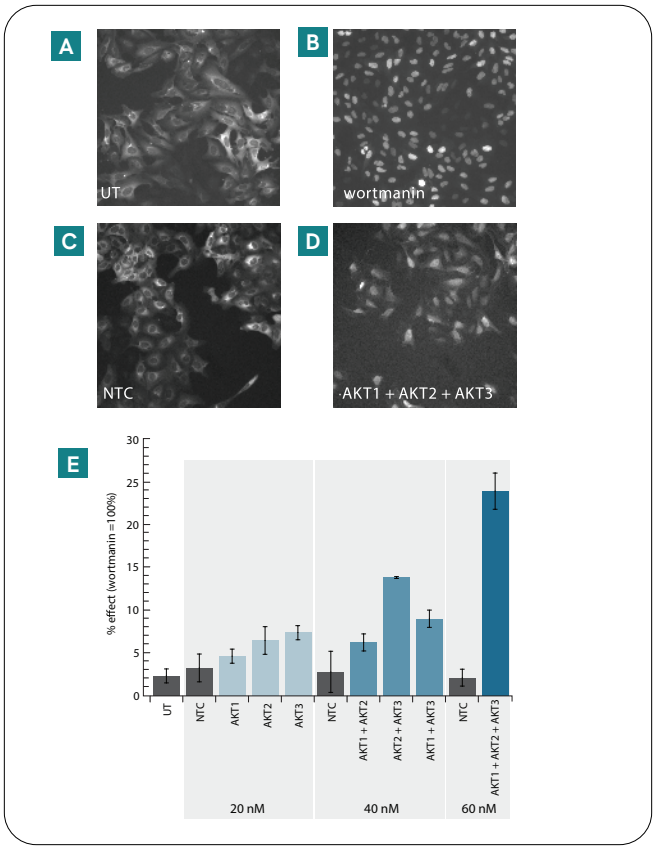

Figure 5: FOXO1 redistribution from cytoplasm to nucleus upon AKT inhibition or down-regulation by siRNAs. **(A-D)** High-content microscopy analysis using the ArrayScan™ VTI HCS Reader was performed on FKHR Redistribution cell line at 72 hours post transfection. Cell images are presented for untreated (UT) samples **(A)**, cells treated with 150 µM wortmannin for 24 hours prior to analysis **(B)**, cells transfected with the Non-Targeting control (NTC) siRNA **(C)**, cells transfected with siRNA pools targeting all three AKT isoforms **(D)**. **(E)** Quantitative analysis of the FOXO1 redistribution for all samples was obtained by the Redistribution™ V3 BioApplication software and is presented as percent of the redistribution effect obtained by the wortmannin control.

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