

RESEARCH PAPER

## BRD4 inhibitor IBET upregulates p27kip/cip protein stability in neuroendocrine tumor cells

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### ABSTRACT

The prevalence of neuroendocrine tumors (NETs) has recently been increasing. Although various drugs such as Octreotide and its analogs show certain efficacy, NETs in many patients progress and metastasize. It is desirable to develop new interventions to improve the therapy. Here we show that human neuroendocrine tumor BON cells are resistant to several drugs commonly used for NET therapy, including Octreotide that activates somatostatin receptor-induced anti-proliferation, and Capecitabine and Temozolimide that damage DNA. In contrast, an inhibitor (IBET) to an epigenetic regulator, Brd4 that binds acetylated histones and upregulates transcription of multiple genes including protooncogene *c-Myc*, potently inhibited the NET cells. We found that IBET increased the protein levels of cyclin-dependent kinase (CDK) inhibitor p27<sup>kip/cip</sup> (or p27), but not its mRNA levels. Moreover, the p27 induction at protein level by IBET was at least partly through increasing the protein stability of p27. The increased protein stability of p27 likely resulted from IBET-mediated suppression of Skp2, an E3 ligase that can mediate p27 degradation by increasing its ubiquitinylation. These findings unravel a new mechanism whereby the IBET-induced repression of proliferation of neuroendocrine cells.

### ARTICLE HISTORY

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### KEYWORDS

Brd4; IBET; p27;  
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### Introduction


Neuroendocrine tumors (NET) not only pose serious threats as neoplastic diseases but also exacerbate the underlying symptoms by secreting an excessive amount of hormones.<sup>1,2</sup> The prevalence of NETs has recently substantially increased.<sup>3</sup> Several organs, such as the pancreas, small intestine, and lungs, can develop carcinoid or neuroendocrine tumors, however, the genetic mechanisms regulating the development and progression of these tumors are not well understood.<sup>4</sup> Several drugs, such as Octreotide and its analogs that mimic somatostatin (SST), bind somatostatin receptors (SSTR), G protein coupled receptors, to suppress protein kinase A (PKA) activity, leading to inhibition of hormone secretion and cell proliferation.<sup>5</sup> While Octreotide and derivatives have been used to attenuate the symptoms of the diseases, the efficacy and specificity of such drugs require improvement.<sup>6</sup> Recently, mTOR (mammalian Target Of Rapamycin) inhibitors such as Everolimus, has also been approved for treatment of neuroendocrine tumors.<sup>7</sup> Other chemotherapy agents such as Capecitabine, a precursor of 5-Fluoro-Uracil, and Temozolimide, a DNA-alkylating agent, have shown some promise in treating patients with metastatic NETs.<sup>8</sup> However, many of these agents fail to suppress growth of NETs after long-term treatment, and many patients eventually succumb to the disease. As such, it is important to explore

more avenues to inhibit NET cells to develop more effective therapy.

It has become increasingly clear that epigenetic regulation plays a very important role in tumorigenesis, maintenance of the cancer phenotype and cancer progression.<sup>9,10</sup> One member of a family of acetylated histone binding proteins, Brd4, can specifically bind the modified histones and the chromatin, facilitating gene transcription. The crystal structure of Brd4 has been solved and it contains a cavity that can specifically bind to the acetylated histones.<sup>11–13</sup> Based on this information, a specific inhibitor, JQ1, was synthesized to block Brd4 binding to the acetylated histones, potently suppressing expression of multiple of proliferative or protooncogenes such as *c-Myc*, leading to tumor suppression.<sup>11–13</sup> Later on, multiple inhibitors targeting Brd4 or some of its homologous proteins were developed, including IBET151, which potentially inhibits transcription of *c-Myc* and CDK4, resulting in potent suppression of leukemia cells.<sup>14</sup>

Brd4 inhibitor was also used to treat the cultured neuroendocrine tumor (NET) cells,<sup>15</sup> such as BON cells, a human neuroendocrine tumor cell line derived from pancreatic NETs.<sup>16</sup> Certain Brd4 inhibitor such as JQ1 can suppress NET cell proliferation, concomitant with reduced expression of *c-Myc* and cyclin D1, both pro-proliferative genes, and increased

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expression of certain anti-proliferative proteins.<sup>15</sup> However, it was unclear that the Brd4 inhibitor also affects expression of tumor suppressors in NET cells.

Cell proliferation or cell cycling are tightly regulated by multiple factors, and a key class of proteins controlling cell cycle are cyclin-dependent kinases (CDKs).<sup>17</sup> CDK4/6 are crucial for controlling G0 or G1 to S phase transition, yet CDK2 is crucial for S to G2 transition. Another class of cell cycle regulators is CDK inhibitors including p27kip/cip (p27 hereafter), which can bind and inhibit several CDKs including CDK4/6 as well as CDK2, thus potently inhibiting proliferation of multiple types of cells.<sup>17</sup>

Expression or function of p27 is compromised in neuroendocrine tumors.<sup>18-20</sup> It is thus conceivable that the expression and function of p27 is tightly controlled by multiple factors. For instance, transcription of p27 is enhanced by anti-proliferative transcription factors such as FOXOs, which can directly bind and activate transcription of p27.<sup>21</sup> Moreover, p27 is also regulated posttranscriptionally. P27 is ubiquitinated by E3 ligase, Skp2, and the ubiquitinated p27 is promptly delivered to proteasomes and is degraded in the proteasomes.<sup>22</sup> p27 is reduced in expression or mutated in a subgroup of NETs, and Brd4 inhibitor JQ1 increased p27 expression in certain NET cells.<sup>15</sup> But it is poorly understood how p27 is regulated in NETs by Brd4 inhibitors. In the current studies, we found that while neuroendocrine tumor BON cells are resistant to several commonly used drugs for NET therapy, they are quite sensitive to a Brd4 inhibitor IBET. Notably, IBET suppresses expression of Skp2, leading to increased stability of p27 and reduced cell proliferation. These findings unravel a new means for Brd4 inhibitors to repress neuroendocrine tumors.

## Results

### **Neuroendocrine tumor (NET) BON cells are resistant to multiple drugs used to treat NETs, but are sensitive to Brd4 inhibitor IBET151 (IBET hereafter)**

To determine the sensitivity of an established human neuroendocrine tumor cell line, BON cells, to the drugs commonly used to treat human NETs, first, we examined the impact of Everolimus,<sup>7</sup> an inhibitor of mTOR kinase which is upregulated in certain human NETs,<sup>23</sup> on BON cells. We found that Everolimus barely reduced cell proliferation in doses ranging from 0.01–100 nM (Fig. 1A), while longer treatment of 5 day slightly reduced cell number (Fig. 2B). We further examined the impact of another commonly used drug for treating NETs, octreotide, which is a synthetic cyclic peptide mimicking the human inhibitory hormone, somatostatin (SST).<sup>24</sup> The results indicate that BON cells were resistant to Octreotide in doses ranging from 0.1–20 μM (Fig. 1B). We also found that Capecitabine and Temozolimide, a nucleotide analog and DNA alkylating agent, respectively, used to treat metastatic NETs,<sup>8</sup> failed to suppress BON cells, in a wide range of doses from 0.005–5 μM of concentrations (Fig. 1C-D). It is likely that BON cells represent a type of metastatic NETs that have developed resistance to multiple drugs.

As such, we explored whether other new drug-like epigenetic inhibitors, such as IBET151 (IBET hereafter), which inhibits an acetylated histone-binding proteins such as Brd4, can affect

growth of the neuroendocrine cells,<sup>14</sup> and a previous report also indicates that this type of inhibitor can suppress certain NET cells.<sup>15</sup> Our results indicate that indeed IBET substantially suppressed growth of BON cells (Fig. 1E). As a control, we also treated these cells with an inhibitor of FOXOs,<sup>25</sup> and found that FOXOi did not suppress growth of BON cells (Fig. 1F).

To further examine the time-dependent effect of these various drugs or drug-like compounds on growth of the NET cells, we monitored the sensitivity of the cells to the various inhibitors in a timeframe up to 5 days, with daily replenishing of the drugs. The cells were treated with the various inhibitors, and counted to determine the impact of the drugs on cell growth. The results indicate that IBET markedly suppressed growth of the NET cells (Fig. 2A), and Everolimus only modestly suppressed growth of the cells after the second day of treatment (Fig. 2B), but the cells were resistant to Capecitabine treatment (Fig. 2C).

Together, our results indicate that BON cells, as a human NET cell line commonly used for cell biology studies, are resistant to multiple commonly used drugs, but that Brd4 inhibitor is effective in inhibiting growth of the cells. However, the underlying mechanism remains unclear.

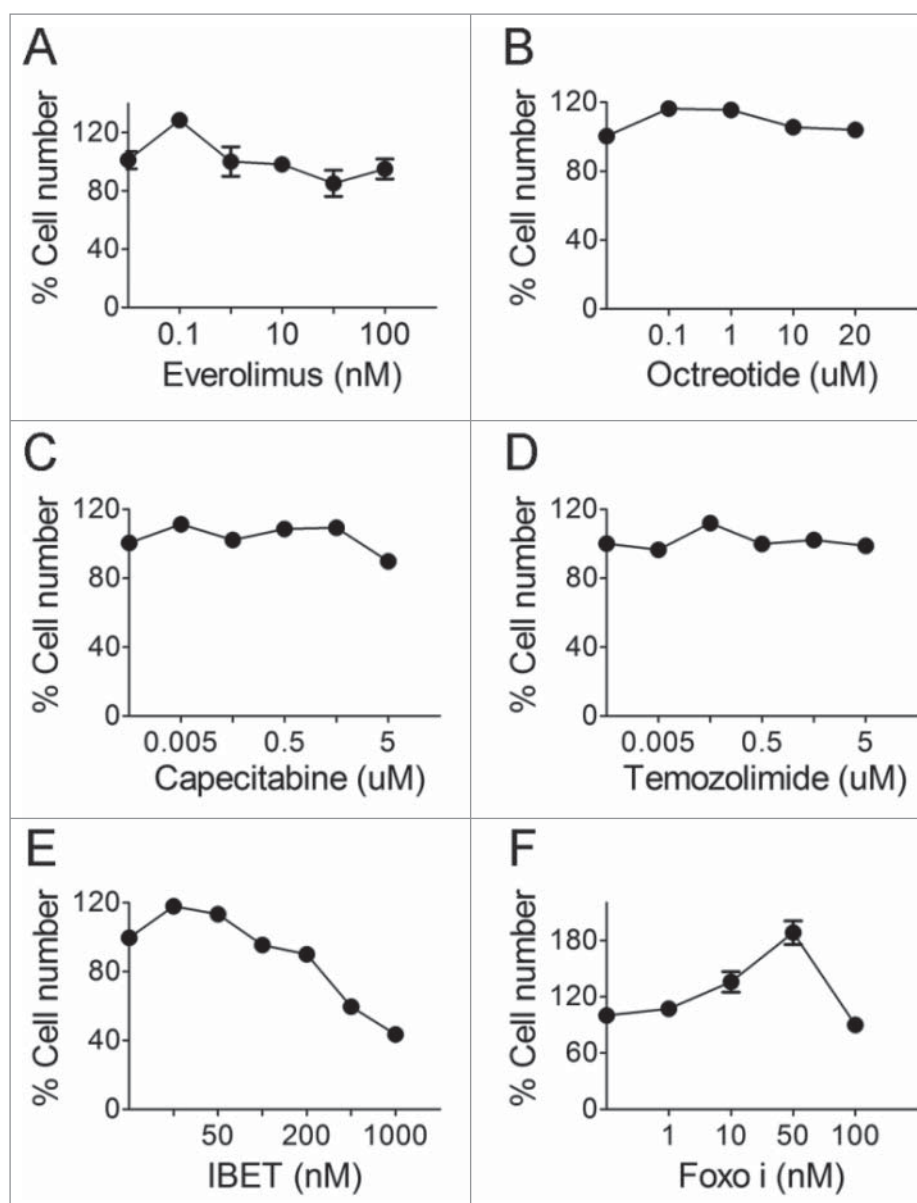
### **IBET induces expression of p27 at the protein level, but does not affect the mRNA level**

To determine how IBET suppressed growth of BON cells, we investigated whether IBET treatment affects various factors that regulate cell proliferation. As it has been reported that IBET decreases expression of cyclin D1<sup>15</sup> and c-Myc,<sup>12</sup> we determined expression of these proteins in the control and IBET treated cells. As expected, IBET treatment reduced expression of cyclin D1 (Fig. S1), and c-Myc (Fig. 3, lane 2), in Western blotting analysis. We further examined whether IBET also affects expression of certain anti-proliferative factors such as p27 and FOXO1. Interestingly, IBET treatment also markedly increased the protein level of p27 (Fig. 3, lane 2), a cyclin dependent kinase inhibitor, and also FOXO1 (Fig. 3, lane 2).

We sought to determine whether IBET-induced expression results from increased mRNA level or transcription of p27 and FOXO1 or not. To this end, we examined the mRNA levels of the control and IBET treated cells using qRT-PCR analysis, and found that IBET treatment slightly increased the mRNA level of FOXO1 (Fig. 4A), but did not affect the mRNA level of p27 (Fig. 4B). Thus far, to our knowledge, the most biologic effect induced by Brd4 inhibitor was mediated by its impact on binding and suppressing Brd4 to the acetylated histones in the chromatin, resulting in suppression of gene transcription.<sup>12</sup> Nevertheless, our findings regarding the impact of IBET on the protein level, but not the mRNA level, of p27 raises an intriguing possibility that IBET induces p27 expression at a posttranscriptional level.

### **Treatment of NET cells with IBET substantially increases the stability of p27**

To determine whether IBET treatment influences the protein stability, we first treated the cells with DMSO or IBET for 3 days, followed by treatment with cycloheximide, an inhibitor of protein translation in cells,<sup>26</sup> for various periods of time before Western blot analysis. The results indicate that in



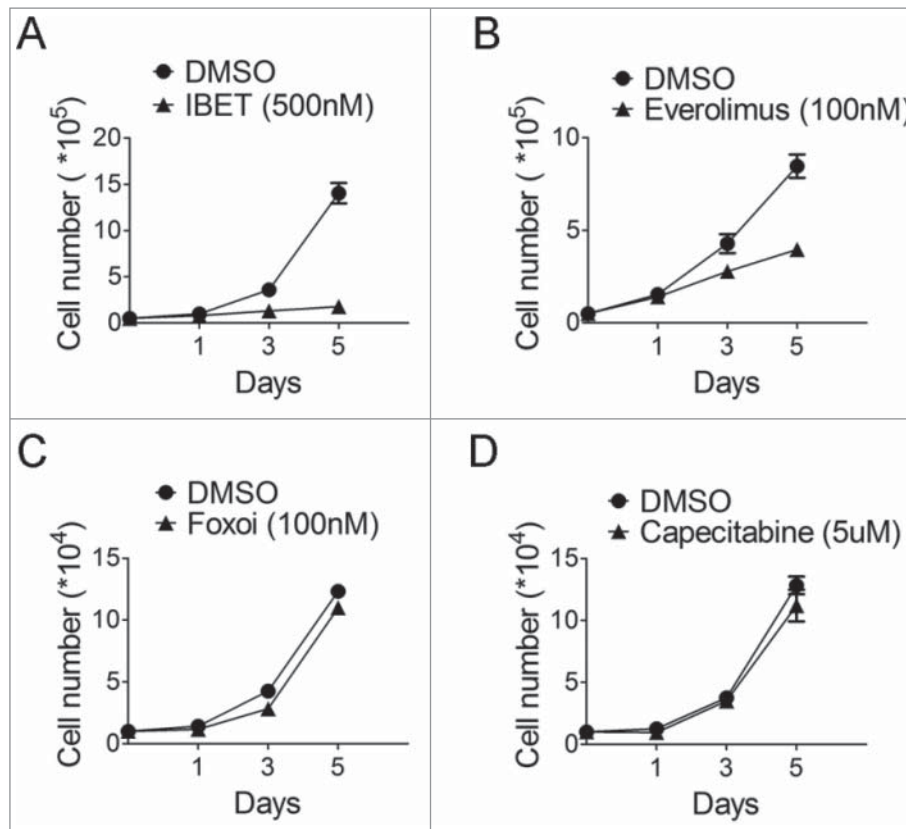
**Figure 1.** The effect of various drugs on BON cell growth. (A-D) BON cells were treated with various concentrations of everolimus (A), octreotide (B), capecitabine (C) and temozolimide (D). MTS assay was performed to assess cell proliferation after 3 d. (E) BON cells were treated with varying concentrations of IBET and MTS assay was performed to assess cell proliferation after 5 d of treatment. (F) BON cells were treated with various concentrations of Foxoi and cell counting was performed to assess cell proliferation after 5 d of the treatment.

control cells, p27 protein degraded quickly 2 hr after the treatment with cycloheximide (Fig. 5A, lane 1 vs 2). In contrast, treatment of the cells with IBET markedly delayed the reduction of p27 protein, even maintaining approximately 60% of the protein after 6 hr of IBET treatment (Fig. 5A, lanes 5–8 and 5B). In control cells, after 6 hr treatment, only less than 20% of p27 was detectable. These results indicate that IBET treatment substantially reduced degradation of p27, an anti-proliferative protein that may contribute to IBET-induced suppression of BON cell growth.

#### **IBET reduces expression of Skp2, an E3 ubiquitin ligase that enhances degradation of p27**

It has been reported that Skp2, an E3 ubiquitin ligase can catalyze protein ubiquitination and promote subsequent

proteasome-mediated degradation.<sup>22</sup> As such, we investigated the potential impact of IBET treatment on the protein level of Skp2. To this end, we treated BON cells with either control vehicle or IBET, and then determined the amount of Skp2 protein using Western blot. The results indicate that indeed treatment with IBET substantially reduced Skp2 protein, in contrast to increased expression of p27 and FOXO1 in protein levels (Fig. 6A, lane 2 vs 1), while the control actin levels remained the same. Moreover, treatment of the BON cells with FOXOi, which blocked IBET-induced increase of p27 protein level (Fig. 3, lane 4), also upregulated the protein level of Skp2 (Fig. 6B). Together, these results indicate that IBET can repress expression of Skp2, leading to increased stability and protein level of p27, thus suppressing NET cell proliferation (Fig. 6C), a novel mechanism whereby IBET inhibits cell proliferation.



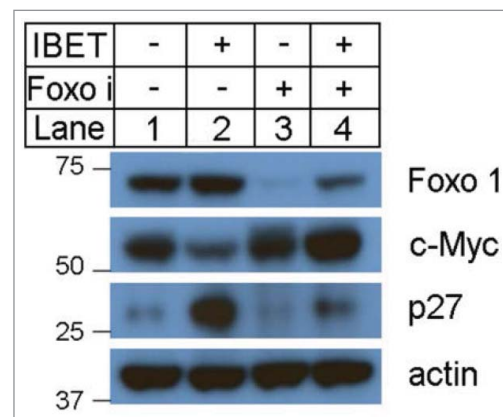
**Figure 2.** The time course of treatment of BON cells with various drugs. (A) Cell counting following treatment of the BON cells with IBET (500nM) for the indicated time. (B) Cell counting following treatment of the BON cells with everolimus (100nM) for the indicated time. (C) Cell counting following treatment of the BON cells with Foxoi (100nM) for the indicated time. (D) Cell counting following treatment of the BON cells with capecitabine (5 $\mu$ M) for the indicated time.

## Discussion

One major challenge in cancer therapy is the development of de novo or acquired resistance.<sup>27</sup> Certain cancer cells develop drug resistance via traceable mutations in the target genes, such as the BCR-ABL kinase mutations in chronic myeloid leukemia (CML) in response to BCR-ABL inhibitor Gleevec.<sup>28</sup> Other secondary mutations in EGFR, whose mutation is a driver in a subset of non-small cell lung cancer (NSCLC), such as T790M that reduces the mutant EGFR binding to its kinase inhibitor in patients, occur in cancer cells, causing drug resistance.<sup>27</sup> On the other hand, the de novo resistance, sometime similar to reduced sensitivity, to drugs of targeted therapy, such as PI3K/AKT inhibitors, takes place by activation of the feedback loop of the kinase pathways such as activation of pro-proliferative receptor tyrosine kinases (RTK).<sup>29</sup> In other cases, the certain adaptive and compensatory pathways are activated epigenetically, in a time dependent manner, resulting reduced drug and failure of therapy. One example is that treatment of human HER2+ breast cancer cells with Lapatinib, a small molecular HER2/HER1 kinase inhibitor, leads to a biphasic response of the cancer cells to the treatment.<sup>30</sup> In the first phase, Lapatinib suppresses HER1/2, eventually leads to inhibition of AKT, reducing the phosphorylation of FOXOs, thus activating FOXOs and cell apoptosis. However, several days later, in the second phase, the remaining cells became quiescent, but markedly upregulating FOXOs, resulting in enhanced transcription of c-Myc via paradoxical FOXO/c-Myc axis and eventual loss of the sensitivity of the HER2+ cancer cells.

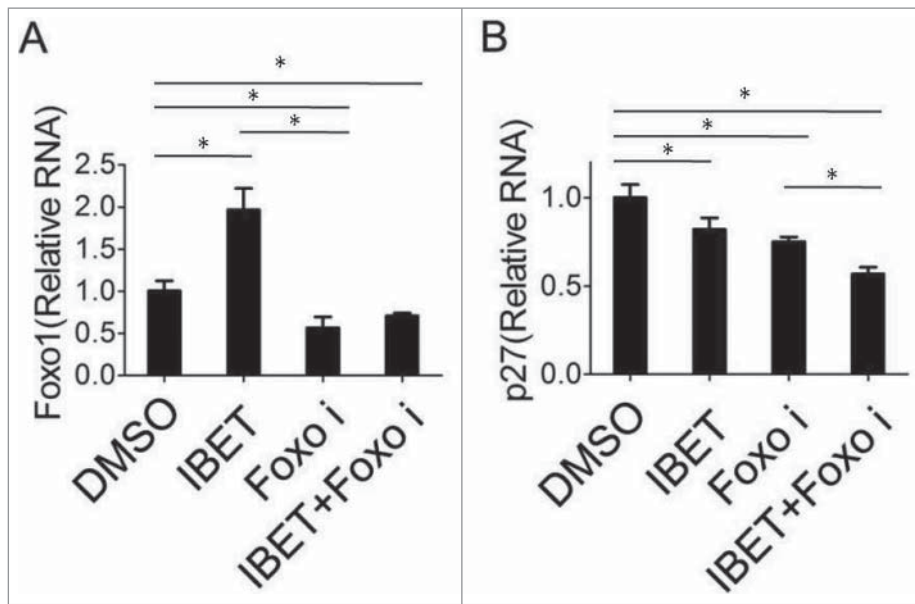
In neuroendocrine tumors, it is even more challenging to uncover the pathways that can cause effective resistance to the drugs as only a few pathways are found to be mutated in the cancer, and no drug resistance-causing mutations in the driver genes have yet been found.<sup>23</sup> As such, we evaluated the impact of the commonly used drugs such as Everolimus, Octreotide, Capacitabine, and Temozolimide on the growth of BON cells. It is not surprising that many of these drugs were not effective to suppress the human NET cells.

IBET can effectively suppress transcription of several key pro-proliferative oncogenes or proto-oncogenes such as c-Myc,



**Figure 3.** The effect of IBET treatment on the protein levels of p27, FOXO1 and c-Myc. The BON cells were treated with control DMSO or IBET (500 nM), for 5 days, followed by Western blotting analysis using the indicated antibodies.





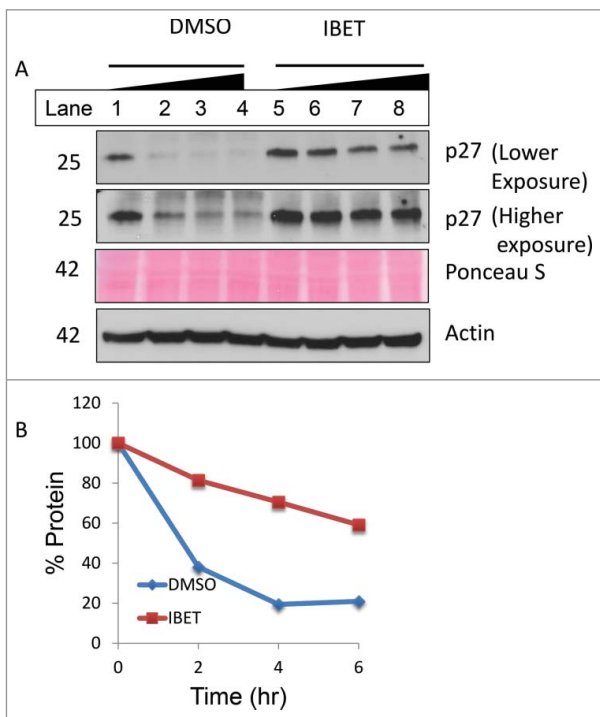
**Figure 4.** The effect of IBET treatment on the mRNA level of p27cip/kip. BON cells were treated with either control DMSO or IBET (500 nM) for 5 days, followed by isolation of RNA from the cells, and qRT-PCR analysis for the FOXO1 and p27 (B). The means of the different groups in the mRNA level were compared using one-way ANOVA test. Differences were considered statistically significant when  $P < 0.05$  (\* $P < 0.05$ ).

CDK4, and cyclin D1, in several types of leukemias or solid tumors.<sup>12,14,15</sup> However, little is known as to how the Brd4 inhibitors affect anti-proliferative genes. Our findings indicate that Brd4 inhibitor not only suppresses cell proliferation via inhibiting pro-proliferative genes, but also coordinately increases anti-proliferative tumor suppressing proteins such as p27. These findings provide new insights into understanding

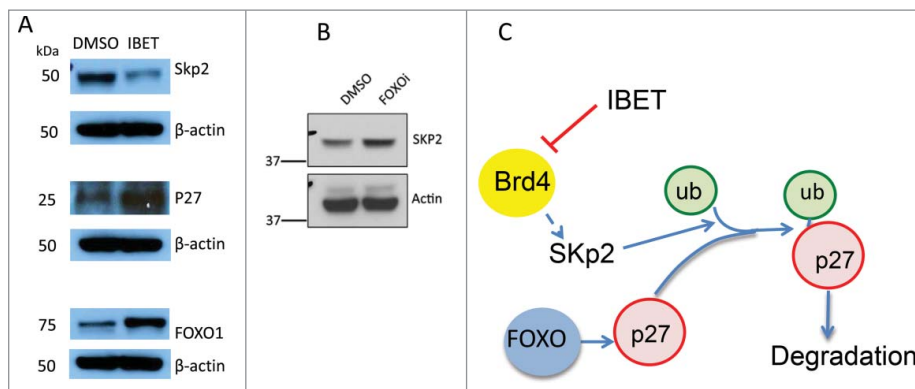
how the Brd4 inhibitor suppresses NET cells via upregulating anti-proliferative proteins.

p27 is regulated by multiple mechanisms including gene transcription induced by FOXOs<sup>21</sup> and protein degradation mediated by Skp2.<sup>22</sup> However, thus far, it is not clear whether IBET affects p27 protein degradation. Our current studies found that IBET increases p27 expression and protein stability at least partly via reducing expression of E3 ligase Skp2, which has been shown to mediate p27 degradation. While FOXOs can increase transcription of p27 in certain cells,<sup>21</sup> our results show IBET can also increase FOXO1 protein as well as mRNA levels, however, it is not yet clear whether IBET-mediated regulation of FOXOs plays any role in regulating p27 stability. On the other hand, we found that IBET increased the p27 protein level but did not affect the mRNA level of p27. IBET treatment markedly increased the stability of p27 in the NET cells. IBET increases the p27 stability and protein level, at least partly via repressing Skp2, which can normally degrade p27 protein by increasing p27 ubiquitinylation, a signal triggering proteasome-mediated protein degradation. Thus, IBET-mediated increase in p27 protein level may contribute to the suppression of proliferation of BON cells.

Consistent with these findings, Brd4 inhibitor JQ1 also upregulates p27 protein in hepatocellular carcinoma cells.<sup>31</sup> Moreover, Brd4 inhibitor also reduced Skp2 but increased expression of p27 protein levels in melanoma cell lines,<sup>32</sup> even though the precise p27 stability was not examined. As it has been reported that Brd4 inhibitor IBET like compound also reduces the mRNA level of Skp2 in melanoma cells,<sup>1</sup> and FOXOs can directly bind to the promoter of the p27 gene to upregulate its expression, it is likely that IBET can inhibit the transcription-activating acetylated histone binding proteins such as Brd4 at the Skp2 promoter to reduce its expression, resulting in reduction of Skp2 but upregulation of p27 protein level. Even at the basal level without marked repression of AKT and upregulation of FOXO activities, it is possible that FOXO $i$  can still reduce the basal



**Figure 5.** IBET treatment increases p27 protein stability. (A) BON cells were treated with DMSO or IBET for 3 days, followed by addition of 20  $\mu$ g/ml cycloheximide in the respective medium, and the cells were collected at 0, 2, 4 and 6 hr after the cycloheximide treatment of Western blotting with the indicated antibodies. (B) p27 protein band in (A) was scanned, and was quantitated using Image J and plotted as % protein relative to the signal value at 0 min.



**Figure 6.** The effect of IBET and FOXO1 treatment on the protein level of Skp2. (A) BON cells were treated with either DMSO or IBET (0.5 μM) for 3 days, followed by Western blotting analysis to detect the protein levels with the indicated antibodies. (B) BON cells were treated with DMSO or FOXO1 (100 nM) for 3 days, followed by Western blotting with the indicated antibodies. (C) A working model to explain IBET-induced upregulation of p27 protein level via suppressing expression of Skp2.

transcription of p27, so that IBET-induced suppression of Skp2 could no longer lead to marked increase in the p27 protein level (Fig. 3, lane 4). Nevertheless, the detailed mechanisms regarding the role of FOXO1 in the process is complex as regulation of the ubiquitin-proteasome system can be mTOR-FOXO1 dependent or independent,<sup>2</sup> and further work is required to elucidate further detailed mechanisms. Together, our findings, coupled with the results from other studies, indicate that IBET mediates suppression of BON cells at least partly by suppressing Skp2 expression and thus leading to increased p27 protein level, contributing to reduced cell proliferation (Fig. 6C).

## Materials and methods

### Cell culture

The human pancreatic neuroendocrine cell lines BON cells were obtained from

Dr. Courtney Townsend from The University of Texas Medical Branch at Gavelston. BON cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 Units/mL penicillin, and 100 mg/mL streptomycin at 37°C in a humidified 5% CO<sub>2</sub>.

### Chemical inhibitors

IBET151 (abbreviated as IBET)<sup>14</sup> was synthesized by Chemizon and purity was >98%. FOXO inhibitor, AS1842856, was purchased from EMD Biosciences. Everolimus was obtained from LC Laboratories (#E-4040). Octreotide was obtained from Sigma (#O-1014). Capecitabine (#SML-0653) and Temozolimide (#T2577) were also purchased from Sigma. IBET, Foxoi and Everolimus were prepared as 10 mM stock solutions in DMSO, and Capecitabine and Temozolimide were prepared as 50 mM stock solutions in DMSO. Octreotide was prepared as 10 mM stock solution in water. All the stock solutions were stored at -20°C.

### Cell proliferation assays

For the MTS assay, BON cells were plated in 96-well plates at a density of  $5 \times 10^3$  cells/well. After overnight incubation, the

cells were treated with either DMSO (control), or various concentrations of Everolimus, Octreotide, Temozolimide or Capecitabine for 72 hr or with IBET for up to 120 hr. The MTS [3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay kit (Promega) was used to assess cell proliferation and was performed according to the manufacturer's instructions. Absorbance of each well was recorded at 490 nm using an ELISA plate reader, and after subtracting a background values, these results were normalized to the value from the control wells. The DMSO treated group was normalized as 100% of proliferation in Fig. 1. Each experiment was performed in triplicate, with mean values  $\pm$  SD reported for each treatment group. For cell counting experiments BON cells were plated in 6-well plates at a density of  $5 \times 10^4$  and  $10^4$  cells/well, respectively. After overnight incubation, the cells were then treated with DMSO (control), IBET, Foxoi or a combination of IBET and Foxoi for 5 d. The attached cells were trypsinized, stained with trypan blue, and the live cells were counted using a hemocytometer. Each experiment was performed in duplicate, with mean values  $\pm$  SD reported for each treatment group.

### Western blotting

BON cells were plated in 10 cm plates at a density of  $1 \times 10^6$  cells/plate respectively and then treated with DMSO (control), IBET or Foxoi, or the combination of IBET and Foxoi. After 5 days, attached cells were collected and then lysed with RIPA buffer containing protease inhibitors. Protein concentrations were determined using a BCA assay kit (Thermo Scientific). Cell lysates were subjected to polyacrylamide gel electrophoresis on Novex gels (Life Technologies), and protein was transferred to PVDF membranes (Life Technologies). Blocking was performed in PBST containing 5% non-fat dry milk.<sup>33</sup> Foxo1 antibody (#2880) was purchased from Cell Signaling Technology. C-Myc (#32072) antibody was purchased from Abcam. P27 (#528) was purchased from Santa Cruz Biotechnology. The antibody for actin (#A5441) was purchased from Sigma. Anti-rabbit and anti-mouse secondary antibodies were purchased from Bio-Rad. The proteins were visualized by detection with Amersham ECL Western blotting detection reagents (GE Healthcare).

## RT-PCR

RNA was extracted from cultured cells with Trizol, and subsequently isolated through the use of an RNeasy Mini Kit (Qiagen). RNA (1.0 µg) was transcribed into cDNA, and real-time PCR (RT-PCR) was performed using a Quantitative SYBR Green PCR Kit (Qiagen) and a 7500 Fast Real Time PCR System (Applied Biosystems).

Foxo1 primers included: forward 5'- CACCATGATGCAGCA GACGC-3' and reverse 5'-CAACTCCTTCAAGCCTCCAG-3'. C-Myc primers included: forward 5'-CTGGTGCTCCAT GAGGAGA-3' and reverse 5'-CCTGCCTCTTTTCCACA GAA-3'.

P27 primers included: forward 5'-GCCCTCCCCAGTCTCTCT TA-3' and reverse 5'-ACACAGCCCCGAAGTGAAAAGA-3'.

Actin primers included: forward 5'-GGTCATCACCATGG CAATGA-3' and reverse 5'-GCACTGTGTTGGCGTACA-3'.

Foxo1, c-Myc and P27 transcript levels were normalized to actin transcript levels, with mean values  $\pm$  SD reported for each group.

## Protein stability assay

BON cells were treated with either DMSO or IBET for 3 days, followed by addition of 20 µg/ml cycloheximide. The resulting cells were collected at 0, 2, 4 and 6 hrs after the treatment of Western blotting with the indicated antibodies. The density of the protein band was quantified and analyzed with Image J program and plotted as % protein relative to the value at 0 min.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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