Biochemical and Biophysical Research Communications 434 (2013) 370–375

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Clock-controlled output gene *Dbp* is a regulator of *Arnt/Hif-1β* gene expression in pancreatic islet β-cells

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ARTICLE INFO

Article history:
Received 24 March 2013
Available online 6 April 2013

Keywords:
DBP
ARNT/HIF-1β
D-box
E4BP4
Circadian rhythm
Pancreatic islet

ABSTRACT

Aryl hydrocarbon receptor nuclear translocator (ARNT)/hypoxia inducible factor-1β (HIF-1β) has emerged as a potential determinant of pancreatic β-cell dysfunction and type 2 diabetes in humans. An 82% reduction in *Arnt* expression was observed in islets from type 2 diabetic donors as compared to non-diabetic donors. However, few regulators of *Arnt* expression have been identified. Meanwhile, disruption of the clock components CLOCK and BMAL1 is known to result in hypoinsulinemia and diabetes, but the molecular details remain unclear. In this study, we identified a novel molecular connection between *Arnt* and two clock-controlled output genes, *albumin D-element binding protein* (*Dbp*) and *E4 binding protein 4* (*E4bp4*).

By conducting gene expression studies using the islets of *Wfs1*$^{-/-}$ *A*^{y}*/a* mice that develop severe diabetes due to β-cell apoptosis, we demonstrated clock-related gene expressions to be altered in the diabetic mice. *Dbp* mRNA decreased by 50%, *E4bp4* mRNA increased by 50%, and *Arnt* mRNA decreased by 30% at Zeitgever Time (ZT) 12. Mouse pancreatic islets exhibited oscillations of clock gene expressions. E4BP4, a D-box negative regulator, oscillated anti-phase to DBP, a D-box positive regulator. We also found low-amplitude circadian expression of *Arnt* mRNA, which peaked at ZT4. Over-expression of DBP raised both mRNA and protein levels of ARNT in HEK293 and MIN6 cell lines. *Arnt* promoter-driven luciferase reporter assay in MIN6 cells revealed that DBP increased *Arnt* promoter activity by 2.5-fold and that E4BP4 competitively inhibited its activation. In addition, on ChIP assay, DBP and E4BP4 directly bound to D-box elements within the *Arnt* promoter in MIN6 cells. These results suggest that in mouse pancreatic islets mRNA expression of *Arnt* fluctuates significantly in a circadian manner and that the down-regulation of *Dbp* and up-regulation *E4bp4* contribute to direct suppression of *Arnt* expression in diabetes.

1. Introduction

Circadian clocks are cell-autonomous molecular oscillators that drive the rhythms of physiology and behavior. Complex circuitries of transcriptional/post-translational regulatory loops allow organisms to coordinate physiological processes in response to environmental changes. In mammals, the central pacemaker is localized in the suprachiasmatic nuclei of the hypothalamus, and is controlled by transcriptional/translational feedback loops involving a set of clock genes. However, many studies indicate that peripheral molecular clocks exist in several organs, including the liver, pancreas, kidney, muscle, and adipose tissue, where feeding/fasting constitute the dominant clock-regulating signals for these tissues [1–5]. Recently, disturbances in the regulation of circadian rhythms have been implicated in the development of metabolic disorders such as obesity and type 2 diabetes [6–8], and mice with circadian clock function abnormalities have been reported to also show abnormal glucose homeostasis [9–11]. The most convincing evidence that clock function within the endocrine pancreas impacts glucose homeostasis has emerged from recent studies in mice with tissue-specific ablation of *Bmal1* [12,13]. Despite normal locomotor activity rhythms, pancreas-specific *Bmal1* knockout mice display more pronounced hyperglycemia than systemic *Clock* mutant or *Bmal1* knockout mice. While these studies have narrowed clock function to the late stage of insulin secretion, the precise molecular details remain to be elucidated.

In peripheral tissues, a number of genes have been found to be regulated downstream from the core clock components, which include transcription factors such as DBP and E4BP4. DBP and E4BP4

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http://dx.doi.org/10.1016/j.bbrc.2013.03.084

are directly regulated by core clock genes, and activate and suppress, respectively, the transcriptional activities of further downstream output genes through the D-box [14]. The contribution of the D-box regulatory loop remains relatively uncharacterized with respect to cell-autonomous circadian oscillations and no islet-specific genes, which have promoter elements interacting with DBP and/or E4BP4, have been determined. A detailed understanding of the roles of DBP and E4BP4 in the circadian rhythms of β-cells and their impacts on the transcriptions of islet-specific genes may provide insights into links between circadian rhythms and diabetes.

ARNT/HIF-1β is a member of bHLH-PAS family and is considered to be an obligate heterodimerization partner for other members of this family, such as HIF-1α, HIF-2α, HIF-3α, and AhR [15]. Gene expression profiling of diabetic human islets revealed that ARNT and its possible target genes are markedly reduced [16]. Diminished glucose-stimulated insulin secretion in islets obtained from β-cell specific *Arnt* deficient mice as well as in *Arnt* knockdown MIN6 cells serve as evidence for the importance of *Arnt* in β-cell function. An elevated glucose concentration, in itself, has been shown to be a negative regulator of ARNT expression in INS-1 (832/13) cells and primary mouse islets [17]. In addition, carbohydrate-responsive element-binding protein (ChREBP) has been shown to bind both directly and glucose dependently to the ARNT promoter in β-cells and to thereby down-regulate its transcription [17]. Investigating other regulators of ARNT should provide new insights into the mechanisms underlying ARNT-mediated β-cell dysfunction.

In this report, we present evidence that ARNT is a direct target of DBP and E4BP4. In the pancreatic islets, *Dbp* and *E4bp4* mRNA displayed robust circadian rhythms. In the islets of mildly obese diabetic *wfs1*$^{-/-}$ *A*^{y}*/a* mice [18], decreased levels of *Dbp* and *Arnt* mRNA were observed while the level of *E4bp4* mRNA was increased. Over-expression of DBP increased the expression of ARNT by transactivating the *Arnt* promoter in cell lines. Furthermore, Chromatin Immunoprecipitation in a pancreatic β-cell line demonstrated that DBP and E4BP4 bound to the *Arnt* promoter region directly *in vivo*. Our results suggest that DBP- and E4BP4-mediated ARNT dysregulation may lead to β-cell dysfunction and diabetes.

2. Material and methods

2.1. Animals

All experimental protocols were approved by the Ethics of Animal Experimentation Committee at Yamaguchi University School of Medicine.

Ten week-old male mice were used for all experiments. The animals were housed in a temperature-controlled (24 °C ± 1 °C) room under a 12-h light: 12-h dark cycle, Zeitgeber time (ZT) 0 was designated as lights on and ZT 12 as lights off. Generation and genotyping of *Wfs1*$^{-/-}$ *A*^{y}*/a* mice were previously described in detail [18].

2.2. Reagents

Mammalian expression constructs for DBP, E4BP4, and REV-ERBα in pCMV-SPORT6 vector were purchased from ATCC (American Type Culture Collection). The RORα or RORγ expression construct was generated by amplifying full-length RORα or the RORγ fragment from MIN6 cDNA and inserting the fragments into the pcDNA3 Vector (Promega, Madison, WI, USA). The *Arnt* luciferase reporter construct was generated by amplifying ~2 kb of the *Arnt* promoter fragment from mouse genomic DNA and inserting this fragment into the pGL3-Basic Vector (Promega).

2.3. Isolation of islets from mice

C56BL/6J and *Wfs1*$^{-/-}$ *A*^{y}*/a* mouse Islets were isolated by ductal collagenase digestion of the pancreas as described previously [19].

2.4. Cell culture and transfection

MIN6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) High Glucose with 15% fetal bovine serum (FBS) at 37 °C under 5% CO_2. Cells were plated in 6-well plates overnight before co-transfection of the *Arnt* promoter luciferase reporter with either LacZ control or clock-related gene expression constructs using Lipofectamine Plus (Life Technologies, Carlsbad, CA, USA). Forty-eight hours post-transfection, cells were assayed for luciferase activity using Dual-Luciferase Reporter Assay Systems (Promega). Luciferase activities were normalized to protein content.

2.5. RNA isolation and real-time RT-PCR

MIN6 cells were transfected with various circadian clock gene expression constructs using Lipofectamine 2000 (Life Technologies). Forty-eight hours post-transfection, cells were harvested and prepared for real-time RT-PCR. Total RNA was extracted from MIN6 cells using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). Total RNA extraction from mouse islets was performed with both Isogen and the RNeasy Mini Kit (Qiagen). cDNA was synthesized with SuperscriptII Reverse Transcriptase (Life Technologies) and subjected to real-time PCR using Power SYBR Green PCR Master Mix (Life Technologies) on an ABI 7300 HT thermal cycler (Life Technologies). The value of each cDNA was calculated using the ΔCt method and normalized to the value of the housekeeping gene, *Gapdh*.

All primers were designed for mouse genes. Primer sequences are listed below.

DBP forward: 5′-CTTTTGACCCTCGGAGACAC-3′
DBP reverse: 5′-ACCTCCGGCTCCAGTACTTC-3′
E4BP4 forward: 5′-GGAGCAGAACCACGATAACC-3′
E4BP4 reverse: 5′-TTCCCCAGTCTTCTTTCAGG-3′
REV-ERBα forward: 5′-CCCTGGACTCCAATAACAACACA-3′
REV-ERBα reverse: 5′-GCCATTGGAGCTGTCACTGTAG-3′
ARNT forward: 5′-AGATTTCACGTCACTCCAACC-3′
ARNT reverse: 5′-GGAAGAACGAGTCTTGGCTGT-3′
GAPDH forward: 5′-AGTATGACTCCACTCACGGCAA-3′
GAPDH reverse: 5′-TCTCGCTCCTGGAAGATGGT-3′

2.6. Immunoblotting

HEK293 cells were maintained in DMEM High Glucose with 10% FBS at 37 °C under 5% CO_2. Cells were transfected with various circadian clock gene expression constructs using Lipofectamine 2000 (Life Technologies). Forty-eight hours post-transfection, cells were lysed for Western Blotting. Ten micrograms of protein samples were separated by SDS–PAGE and transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). The membranes were incubated with antibodies against ARNT (Becton Dickinson, Franklin Lakes, NJ, USA), E4BP4 (Abcam, Cambridgs, UK), DBP (AVIVA Systems Biology, San Diego, CA), REV-ERBα (Abnova, Taipei City, Taiwan) and αTubulin (Sigma–Aldrich, St.Louis, MO, USA).

2.7. Chromatin immunoprecipitation analysis (ChIP)

MIN6 cells were cultured in a 10-cm dish until reaching 80% confluence. Cells were fixed with 1% formaldehyde. An EZ-ChIP chromatin immunoprecipitation kit (Merck Millipore, Billerica,

MA, USA) was used to perform the ChIP assay. Cells were lysed and sonicated. Immunoprecipitation was conducted at 4 °C overnight. The ChIP reactions contained the following antibodies: rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA), acetyl histone H3 (Merck Millipore), E4BP4 (Abcam), DBP (AVIVA Systems Biology), REV-ERBα (Abnova), RORα (Abcam), and RORγ (Abcam). ChIP reactions were washed and chromatin was eluted, according to the manufacturer's instructions. Chromatin was purified using PCR clean up columns (Qiagen). PCR was performed using AmpliTaq Gold PCR Master Mix (Life Technologies).

All primers were designed for mouse genes. Primer sequences are listed below.

−2052/−1653 forward: 5′- AATTGTGTTCCAATACTCAGGTG-3′
−2052/−1653 reverse: 5′- CCTAACATCCCAAGATCCAATG-3′
−1576/−1187 forward: 5′- CAGACTGACAACAAGCCAAAG-3′
−1576/−1187 reverse: 5′- ACTCAGGAGGCAGAGACTGG-3′
−1021/−604 forward: 5′- GACCTCTGCTTGCTCCAATC-3′
−1021/−604 reverse: 5′- CCTCCAAAAGTGAGGTGTCC-3′
Mouse PCK forward: 5′- GAGTGACACCTCACAGCTGTGG-3′
Mouse PCK reverse: 5′- GGCAGGCCTTTGGATCATAGCC-3′

2.8. Statistical analysis

Results are expressed as means ± SE. Differences between means were evaluated using Student's *t* test as appropriate. The statistical significance of differences among more than two groups was analyzed by ANOVA and Tukey's post hoc comparisons. A 5% level of probability was considered significant.

3. Results

Wfs1$^{-/-}$ mouse β-cells are susceptible to ER stress. When ER stress is evoked in β-cells by introducing systemic insulin resistance (*Wfs1*$^{-/-}$ *A*^{y}*/a* mice), these mice develop selective β-cell loss and severe insulin-deficient diabetes as early as 8 weeks of age [18]. This β-cell loss is attributable to apoptosis. At the same time, glucose-stimulated insulin secretion is markedly impaired in β-cells from *Wfs1*$^{-/-}$ mice. However, the precise molecular details of pancreatic β-cell dysfunction in *Wfs1*$^{-/-}$ *A*^{y}*/a* remain unknown. To elucidate the mechanism of β-cell dysfunction in *Wfs1*$^{-/-}$ *Ay/a*, we performed microarray analyses. In the islets of *Wfs1*$^{-/-}$ *A*^{y}*/a*, *Dbp* was decreased and *E4bp4* was increased when compared to *Wfs1*$^{+/+}$ *A*^{y}*/a* islets (data not shown). The microarray results indicate the possible involvement of *Dbp* and *E4bp4* in β-cell dysfunction in *Wfs1*$^{-/-}$ *A*^{y}*/a*. *Dbp* and *E4bp4* are known to be clock output genes. Therefore, we examined the circadian oscillations of *Dbp* and *E4bp4* in mouse pancreatic islets. C57BL/6J mice, kept under a light–dark regimen, were killed at 4-h intervals to prepare whole-cell mRNA from isolated islets. The relative expression levels of the mRNA in isolated islets were quantified by real-time RT-PCR. *Dbp*, *E4bp4* and *Rev-erbα* mRNA displayed robust circadian rhythms (Fig. 1A). The highest levels of *Dbp* and *E4bp4* mRNA were observed at ZT12 and ZT0, and exhibited marked fluctuation with a 35-fold and a 10-fold peak/trough ratio, respectively. The rhythm ratio was 27-fold for *Rev-erbα*, an important metabolic regulator, expressed in a circadian manner in tissues such as the liver, adipose tissue, muscle, and the pancreas (Fig. 1A). The structural similarity of ARNT to core clock genes, BMAL1 and CLOCK, prompted us to speculate that ARNT is a circadian clock-controlled gene. The expression level of *Arnt* mRNA modestly, but significantly (rhythm ratio 1.5-fold), fluctuated in a circadian manner with a peak at ZT4 in pancreatic islets from wild-type mice (Fig. 1A).

We analyzed clock-related genes and *Arnt* expression levels in islets of *Wfs1*$^{-/-}$ *A*^{y}*/a* at the dark-to-light (ZT0) and light-to-dark (ZT12) transitions by real-time RT-PCR. At ZT0, *Dbp*, *Rev-erbα*, and *Arnt* mRNA levels decreased by 0.4-, 0.3-, and 0.5-fold, respectively, in the *Wfs1*$^{-/-}$ *A*^{y}*/a* islets (Fig. 1B). At ZT12, *Dbp*, *Rev-erbα*, and *Arnt* mRNA levels significantly decreased by 0.4-, 0.5-, and 0.8-fold, respectively, in the *Wfs1*$^{-/-}$ *A*^{y}*/a* islets (Fig. 1B). At ZT12, the *E4bp4* mRNA level was significantly increased, by 1.6-fold, in the *Wfs1*$^{-/-}$ *A*^{y}*/a* islets (Fig. 1B).

We then examined the effects of clock-related gene over-expressions on *Arnt* expression in vitro to investigate the molecular mechanisms underlying our observations in *Wfs1*$^{-/-}$ *A*^{y}*/a* mice. The endogenous ARNT protein expression level significantly increased, approximately doubling, in HEK293 cells transiently transfected with the DBP over-expression plasmid versus the control plasmid (Fig. 2A and B). The expression level of endogenous *Arnt* mRNA significantly increased, by approximately 1.2-fold, in MIN6 cells transiently transfected with the DBP over-expression plasmid versus the control plasmid (Fig. 2C). Neither E4BP4 nor REV-ERBα over-expression affected *Arnt* mRNA and protein levels in either cell line (Fig. 2).

To further examine direct regulation of the *Arnt* gene by clock-related genes, the luciferase reporter construct was co-transfected with clock-related protein expression vectors in MIN6 cells. The reporter construct was composed of a ∼2 kb fragment of the *Arnt* promoter that contains the putative D-box sites (−1932 to −1912 and −1702 to −1691) and the RORE site (−1519 to −1507). Relative luciferase activity was calculated based on the value of the LacZ group set as 1. DBP over-expression increased *Arnt* promoter activity by 2.5-fold. E4BP4 and REV-ERBα abolished activation of the *Arnt* promoter by DBP. *Arnt* promoter activity was unresponsive to either RORα or RORγ (Fig. 3). These data confirmed both D-box sites and the RORE site within the *Arnt* promoter to be functional elements.

The ChIP assay was performed in MIN6 cells to examine whether clock-related genes were recruited to the *Arnt* promoter in pancreatic β-cells. We used TFSEARCH to examine the *Arnt* promoter and indeed located two putative D-box sites (D1: −1932 to −1912 and D2: −1702 to −1691) and the RORE site (−1519 to −1507) as shown in Fig. 4A. The locations of ChIP primers are also presented (Fig. 4A). Employing ChIP analyses, we identified selective recruitments of DBP and E4BP4 to the D-box sites, and REV-ERVα, RORα, and RORγ to RORE site, as compared to the IgG and mouse phosphoenolpyruvate carboxykinase (PCK) promoter controls (Fig. 4B).

4. Discussion

Recent studies in humans and rodents have demonstrated that clock genes are closely linked to obesity and diabetes [6–11]. For instance, *Clock* and *Bmal1* mutant islets show impaired insulin release in response to glucose [12,13]. Our results demonstrate that the *Arnt* gene is a newly-recognized direct target gene of DBP, a clock-controlled output gene, and may serve as a key player in connecting circadian rhythm abnormalities and pancreatic β-cell dysfunction.

The *Dbp* mRNA expression level is reportedly decreased in the hepatic and adipose tissues of obese, diabetic ob/ob mice [20]. However, daily mRNA expression profiles of pancreatic islets have not yet been analyzed in diabetic mouse models including ob/ob mice. Thus, *Wfs1*$^{-/-}$*A*^{y}*/a* could be the first model in which altered expression levels of clock output genes were demonstrated in pancreatic islets.

Richardson et al. reported the diurnal changes in ARNT proteins in multiple tissues from female Sprague–Dawley rats [21]. In their study, two expression peaks were demonstrated at ZT5 and ZT22 in the liver and lungs. In contrast, no apparent daily cycle of ARNT

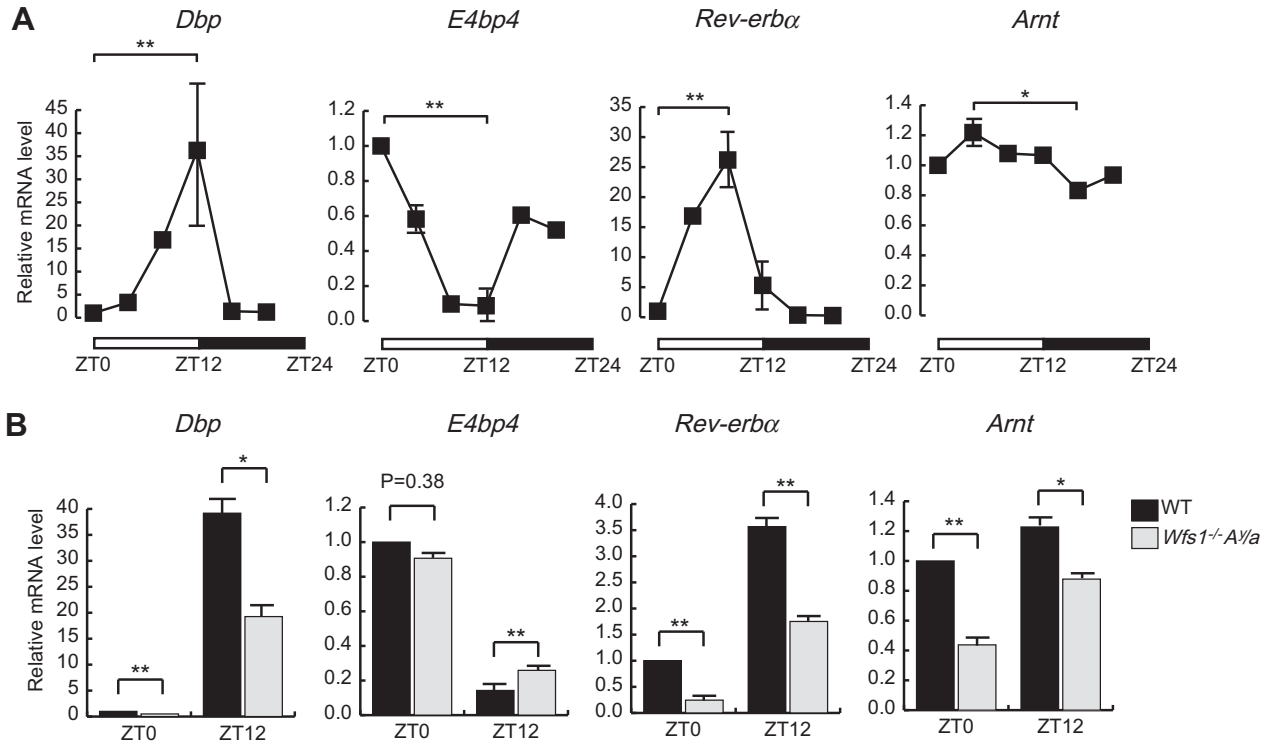

Fig. 1. Circadian expressions of *Dbp*, *E4bp4*, *Rev-erbα* and *Arnt* in isolated islets are shown. The mRNA relative expression levels in isolated islets were quantified by real-time RT-PCR (lights on ZT0, lights off ZT12). (A) Oscillations of *Dbp*, *E4bp4*, *Rev-erbα* and *Arnt* in islets of C57BL/6J mice (mean ± SE, $n = 3$ mice per time point, $^{*}P < 0.05$; $^{**}P < 0.01$). *Dbp* cycled reaching its zenith around ZT12 and its nadir at ZT0. *E4bp4* cycled anti-phase to *Dbp*. *Rev-erbα* cycled reaching its zenith around ZT10 and its nadir ZT0. *Arnt* mRNA fluctuated markedly in a circadian manner with a peak at ZT4. The rhythm ratio was 1.5-fold. (B) The expression levels of *Arnt*, *Dbp* and *Rev-erbα* were significantly decreased in the *Wfs1*$^{-/-}$ *A*^{y}*/a* islets at ZT0 and ZT12. At ZT0, the *E4bp4* expression levels did not differ between *Wfs1*$^{-/-}$ *A*^{y}*/a* and control islets. At ZT12, the *E4bp4* expression level was significantly increased in the *Wfs1*$^{-/-}$ *A*^{y}*/a* islets (mean ± SE, $n = 3$–4 per time point, $^{*}P < 0.05$; $^{**}P < 0.01$).

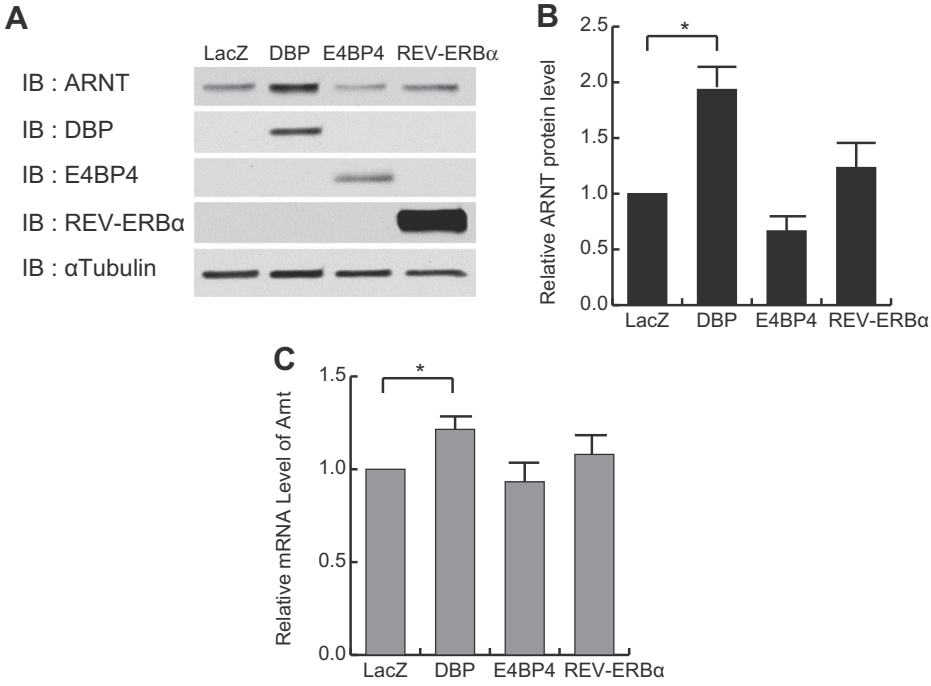

Fig. 2. ARNT expression was increased by DBP over-expression in HEK293 cells and MIN6 cells. (A and B) HEK293 cells were transfected with clock-related gene expression plasmids. Western blot analysis demonstrated the relative protein level of ARNT to be significantly increased by DBP over-expression (mean ± SEM, $n = 4$, $^{*}P < 0.05$). Neither E4BP4 nor REV-ERBα over-expression changed the ARNT expression level. Over-expressions of DBP, E4BP4, and REV-ERBα, using their respective over-expression plasmids, were confirmed. (C) MIN6 cells were transfected with clock-related gene expression plasmid, and the *Arnt* mRNA level was analyzed by real-time RT-PCR. *Arnt* mRNA was significantly increased only by DBP overexpression (mean ± SE, $n = 3$, $^{*}P < 0.05$).

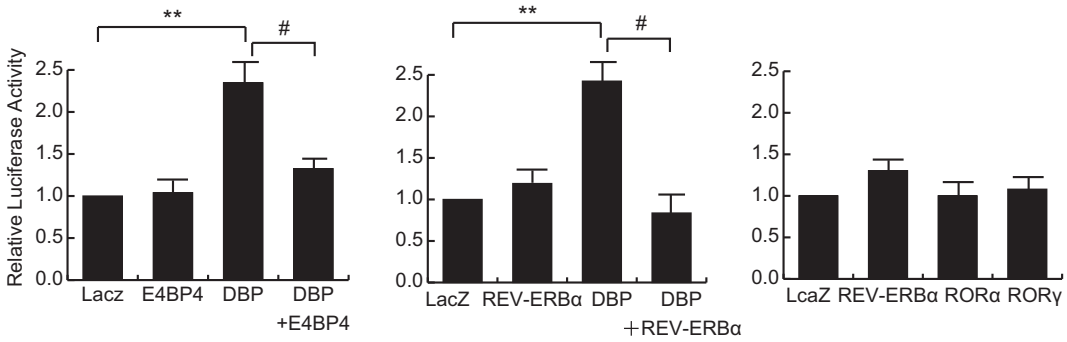

Fig. 3. Transactivation of the *Arnt* promoter by clock-related genes is shown. A mouse *Arnt* promoter-driven luciferase vector was co-transfected with the expression vectors encoding clock-related genes in MIN6 cells. The relative luciferase activities were calculated based on the value of the LacZ group set as 1. DBP over-expression activated the *Arnt* promoter by 2.5-fold. E4BP4 and REV-ERBα abolished this *Arnt* promoter activation by DBP ($n = 3$, mean ± SE, $^{**}P < 0.01$, $^{\#}P < 0.05$). Neither RORα nor RORγ activated the *Arnt*-luc.

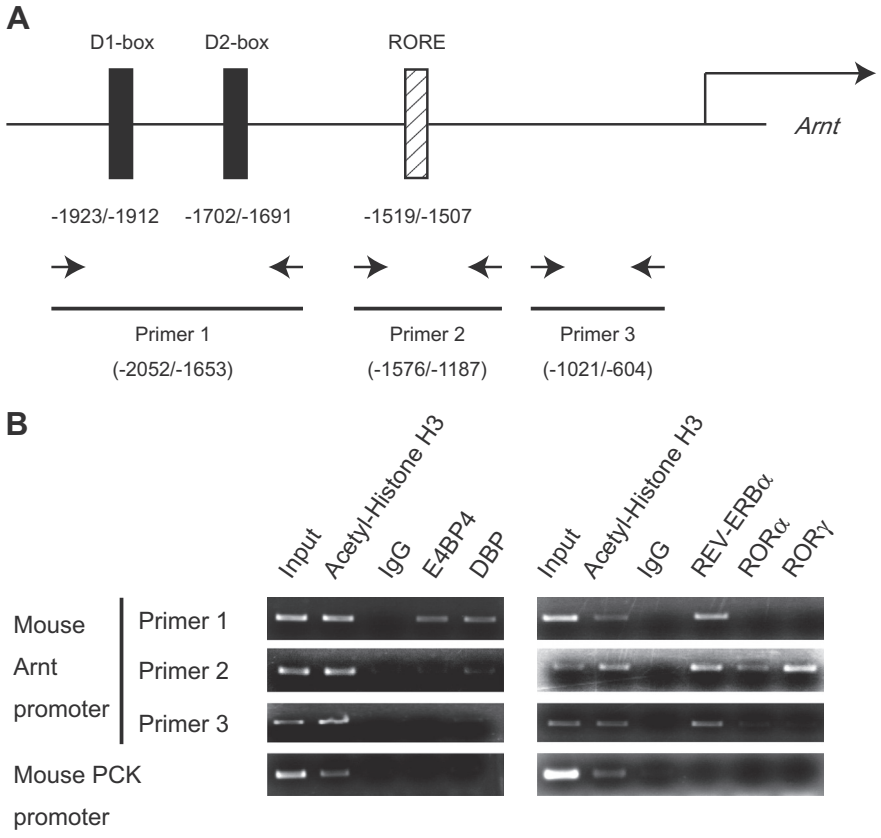

Fig. 4. Clock-related genes bound to the *Arnt* promoter *in vivo*. ChIP was performed to detect DBP, E4BP4, REV-ERBα, ROR-α, and ROR-γ binding to the mouse *Arnt* promoter in MIN6 cells. (A) Schematic diagram shows the putative binding sites (D1-box, D2-box, and RORE) within the 2 kb upstream of the transcription start site. Locations of the ChIP primers are also shown. (B) Endogenous levels of DBP and E4BP4 can be detected at the mouse *Arnt* promoter region containing the identified putative D-box sites. Endogenous levels of REV-ERBα, RORα, and RORγ can be detected at the mouse *Arnt* promoter region containing the identified putative RORE site as well. IgG serves as a negative control, whereas the input and acetyl-Histone H3 serve as positive controls. As a ChIP negative control, amplification of a promoter region of phosphoenolpyruvate carboxykinase (PCK) was included in the experiment. Only the DNA input and acetyl-Histone H3 showed amplification (B, the lowest panel).

protein expression is observed in the spleen. These data also suggest tissue-specific differences in ARNT protein oscillation. The daily cycle of ARNT expression in the pancreas has not yet been reported. In our present study, the daily cycle of *Arnt* mRNA expression was significant in mouse pancreatic islets, suggesting that *Arnt* expression is at least partly governed by clock genes (Fig. 1A). If *Arnt* expression were governable only by DBP and E4BP4 among circadian related genes, *Arnt* should display a robust circadian rhythm with high amplitude. In fact, the amplitude of circadian *Arnt* oscillation was obviously lower than those of clock genes. *Dbp*-dependent circadian transactivation of the *Arnt* gene appears to be exerted via not only the D-box but also other enhancer elements including RORE interacting with REV-ERBα. REV-ERBα, oscillating in the same phase as DBP, may negatively regulate and fine-tune *Arnt* expression.

Few studies to date have investigated key regulators of ARNT in pancreatic β cells [17,22]. Nordeen et al. first confirmed that ChREBP bound to the *Arnt* promoter in a glucose concentration-dependent manner, and decreased ARNT expression levels in INS-1 (823/13) cells [17]. These in vitro studies may indicate that hyperglycemia

suppresses ARNT expression *in vivo*. Gunton et al. examined *Arnt* expression in isolated islets from ob/ob mice, db/db mice, and β-cell -specific insulin receptor knockout mice (βIRKO). The first two models had hyperglycemia, but showed no significant changes in *Arnt* expression [16]. *Wfs1*$^{-/-}$*A*^{y}*/a* mice also had hyperglycemia (363 mg/dl versus 204 mg/dl in controls) at 10 weeks of age. These *in vivo* data do not suggest that a substantial decrease in *Arnt* expression is likely to be mediated solely by hyperglycemia *in vivo*.

Dror et al. showed that blocking Ca^{2+} flux through the ryanodine receptor (RyR) increased the expressions of ARNT and presenilin-1 in pancreatic β-cells [22]. In addition, they also found that ARNT is downstream from presenilin-1 [22]. WFS1 functions as an endoplasmic reticulum (ER) calcium channel or as a regulator of ER calcium channel activity and the defective insulin secretion characteristic of *Wfs1*$^{-/-}$ mice is accompanied by a reduced cellular calcium response [19,23]. Given that blocking intracellular calcium ion release channels can induce the expression of ARNT via presenilin-1 up-regulation, an imbalance in ER homeostasis caused by inactivation of WFS1 may have reduced *Arnt* expression.

In addition to the involvement of WFS1, we tested the possibility that DBP and E4BP4 directly regulate ARNT expression. Overexpression of DBP increased ARNT expression in HEK293 cells and MIN6 cells (Fig. 2A and B). In MIN6 cells, the effect was weaker perhaps due to high expressions of endogenous ARNT and DBP. Furthermore, transient transfection/reporter gene assay results suggested that all three circadian-responsive elements were functional in MIN6 cells (Fig. 3). E4BP4 or REV-ERBα abolished the *Arnt* promoter activation induced by DBP, but not basal promoter activation (Fig. 3). These findings suggest that, in β-cells, *Arnt* promoter activity needs to be at the basal level to maintain β-cell function. Furthermore, we identified DBP occupancy of the *Arnt* promoter and therefore confirmed *Arnt* as a direct target of DBP (Fig. 4).

Clock mutant islets exhibit significant alterations in the expressions of genes involved in post-transcriptional modification and protein packaging [12,22]. While prior studies have narrowed clock function to the late stage of insulin secretion, the precise molecular details remain to be elucidated. *Clock* mutant islets also show decreased expression of *Dbp* during the light cycle [12]. Given our findings, the impaired insulin secretion observed in *Clock* mutant β-cells could in part be a consequence of decreased DBP expression followed by decreased ARNT expression.

In summary, we uncovered a transcriptional pathway linking ARNT, a critical metabolic regulator, with the circadian clock through a circadian-controlled transcription factor, DBP. Our findings point toward β-cell function as operating downstream from the clock network, opening the possibility that the transcriptional pathway linking ARNT with DBP may provide promising new targets for pharmacological control of glucose metabolism.

Acknowledgments

This study was supported in part by Grants-in-Aid for Scientific Research (Grant Nos. 20390093 and 23390080 to Y. Tanizawa) from the Ministry of Education, Culture, Sports, and Science, Grant 10103462 (to Y. Tanizawa) from the Ministry of Health, Labor and Welfare of Japan, and a research grant from Banyu Life Science Foundation International (to K. Tanabe). The authors thank members of their division for helpful discussions.

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