1   **NR4A3 is oxidative stress responsible transcription factor**

2   **through HMOX1, and also controls cell cycle through CCNE1**

3   **and CDK2 in pancreatic islet derived 1.1B4 cells**

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1   ABSTRACT

2   The mechanism of antioxidant defense system is still controversial. As islet ß-cell is weak in

3   oxidative condition, that causes diabetes mellitus, therefore, antioxidant defense system of

4   human pancreatic islet derived 1.1B4 cell was analyzed. Cells were exposed to H_2O_2 and

5   comprehensive gene expression was analyzed by Agilent human microarray. *HMOX1* and

6   *NR4A3*, member of orphan receptor, were up-regulated. Therefore, *NR4A3* was knocked down

7   with siRNA, then analyzed gene expression by microarray, and found that the knocked down

8   cells were weak in oxidative stress. *HMOX1* expression was strongly inhibited by siRNA of

9   *NR4A3*, and *NR4A3* responsible sequence of aaggtca was found near the *HMOX1* gene,

10   suggesting *NR4A3* is oxidative stress responsible transcription factor through *HMOX1*

11   expression. The expression of *CCNE1* and *CDK2* was also inhibited by knocked down of

12   *NR4A3*, it is suggested *NR4A3* is also important transcription factor for cell growth regulation.

13   [Supplemental material is available for this article.]

14

15   **INTRODUCTION**

16   The relentless decline in ß-cell function frequently observed in type 2 diabetic patients and loss

17   of functional ß-cell mass is a hallmark of type 1 and type 2 diabetes, and methods for restoring

18   these cells are needed (Tessem et al. 2014). However, despite optimal drug management, loss of

19   ß-cell is not possible to stop, and has variously been attributed to glucose toxicity and lipo-

20   toxicity. The former theory posits hyperglycemia, that elevated glucose concentrations increase

21   levels of reactive oxygen species (ROS) in ß-cell (Robertson 2004), which takes place within

22   multiple mitochondrial and non-mitochondrial pathways. For example, high concentrations of

23   glucose in vitro increase intracellular peroxide levels in islets, and decrease insulin expression

24   by decreasing *PDX-1* and *MafA* expression (Robertson and Harmon 2006). In their paper, six

2

1   biochemical pathways along which glucose metabolism suggested to form ROS. One of

2   pathway is glycation that is hyperglycemia produces glycation end product (AGE) that increases

3   ROS, as AGE-BSA is known to increase production of ROS, and its apoptogenic effect was

4   blocked by the antioxidant N-acetylcysteine (Weinberg et al. 2014). In physiologic

5   concentrations, endogenous ROS help to maintain homeostasis, however, when ROS

6   accumulate in excess for prolonged periods of time, they cause chronic oxidative stress and

7   adverse effects. Thus, increased glucose produces ROS, and pancreatic ß-cells are more

8   sensitive to oxidative stress than other cell types because the expressions of antioxidant

9   enzymes are low levels in pancreatic ß-cell (Robertson 2004). Therefore, oxidative stress leads

10 cellular damage to ß-cell dysfunction. The effect of oxidative stress, however, on the ß-cell is

11 not understood well. Nitric oxide, peroxynitrite, hydrogen peroxide and other oxygen-reactive

12 species might be involved in ß-cell destruction during diabetes development (Mathis et al. 2001).

13 Oxidative stress induces gene expression in heme oxygenase 1 (HMOX1), and the HMOX1

14 gene (*HMOX1*) is frequently activated under a variety of cellular stress conditions, by four

15 pathways including heat-shock factor, nuclear factor-kappa B, nuclear factor E2-related factor 2

16 (NRF2), and activator protein-1 families, are arguably the most important regulators of the

17 cellular stress response in vertebrates (Alam and Cook 2007). One of them, the Kelch-Like

18 ECH-Associated Protein 1 (KEAP1)-NRF2 pathway in which pathway, NRF2 transcription

19 factor directly bind to antioxidant responsible element (ARE) to induce antioxidant enzymes

20 (Nguyen et al. 2009). However, another unknown pathway may be involved. For example,

21 chronically excessive glucose and ROS levels can cause decreased insulin gene expression via

22 loss of the transcription factors pancreatic and duodenal homeobox 1 (PDX1) and v-maf avian

23 musculoaponeurotic fibrosarcoma oncogene homolog A (MAFA) and can also accelerate rates

24 of apoptosis (Robertson 2004).

25   High glucose and oxidative stress induce cell damage not only to ß-cell but also to other cells.

26 There are many transcription factors and sensing proteins. Oxidants such as H_2O_2 can damage

1   proteins, regulate transcription factors, and sometimes induces cell apoptosis (Marinho et al.

2   2014). In his review, the regulatory mechanisms by which H_2O_2 modulates the activity of

3   transcription factors in bacteria (*OxyR* and *PerR*), lower eukaryotes (*Yap1, Maf1, Hsf1* and

4   *Msn2/4*) and mammalian cells (*AP-1, NRF2, CREB, HSF1, HIF-1, TP53, NF-κB, NOTCH, SP1*

5   and *SCREB-1*) are summarized. Furthermore, Klamt *et al*. (Klamt et al. 2009) found that the

6   actin-binding protein cofilin is a key target of oxidation. When oxidation of this single

7   regulatory protein is prevented, oxidant-induced apoptosis is inhibited.

8   As described above, high glucose increases ROS, and antioxidants repairs ß-cells

9   undergoing damage by oxidative stress, however, molecular mechanism of ROS on the islet ß-

10   cell is not well understood. Therefore, in this paper we focus on the mechanism of gene

11   expression by ROS using H_2O_2 in human pancreatic ß-cells derived hybrid cell. Because,

12   transformed cell used for in vitro experiment, the cells sometime lost their characteristic

13   especially apoptosis pathway, otherwise cell will die. Furthermore, human normal islet cells are

14   not available easily, 1.1B4 cells, the hybrid cell of normal islet cell with ß-cell derived

15   transformed PANC-1 cell was used here (McCluskey et al. 2011). In this paper we analyzed the

16   effect of H_2O_2 on the gene expression of 1.1B4 with Agilent microarray. Then key protein was

17   knock down by siRNA method and analyzed cell viability under the H_2O_2 addition. And gene

18   expression of siRNA treated cells was analyzed by microarray again to find how this knock

19   down of the key protein effect gene expression.

20

21   **RESULTS**

22

23   **Effect of H_2O_2 on 1.1B4 cells growth and gene expression analyzed by DNA microarray**

24   Human pancreatic islet derived 1.1B4 cells were exposed to 10, 20, 30, 40, 50, 100, 200 μM

25   H_2O_2, and the cell viability was measured by MTT assay (Fig. 1). We found that there was no

26   toxicity of H_2O_2 up to 50 μM, and the cell growth was inhibited at 100 μM of H_2O_2 and

27   completely suppressed at 200 μM. From this result, it was suggested that even the 1.1B4 cells

28   express antioxidant enzymes to survive from H_2O_2 stress but at higher concentration above 50

29   μM of H_2O_2, cells were not able to grow well because of not enough antioxidant defense system.

30   Therefore, to find antioxidant system of these cells, comprehensive analysis of gene expression

31   of H_2O_2 treated 1.1B4 cells was analyzed by DNA microarray at 100 μM H_2O_2. The 1.1B4 cells
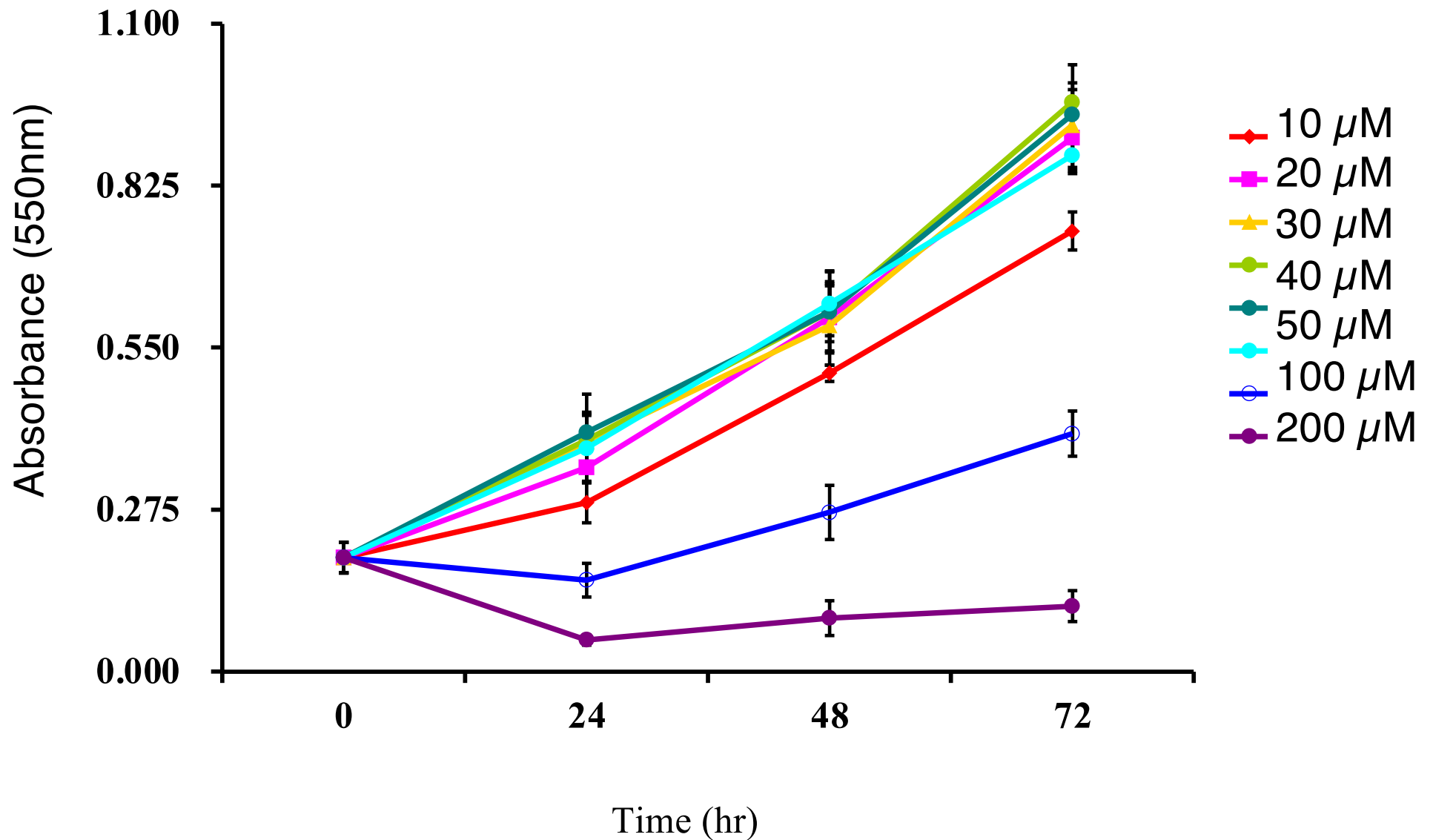
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Fig.1. Effect of H_2O_2 concentration on the cell growth of 1.1B4 islet derived cell. Islet derived 1.1B4 cell was seeded and cultured in RPMI-1640 medium, and 24 hours later cell was added H_2O_2 to 10, 20, 30, 40, 50, 100, 200 μM and cell viability was analyzed at 24, 48 and 72 hours by MTT assay. (n=5)

Table 1. Comparison of expression of genes measured by microarray and real time PCR between H2O2 treated cells and control cells.

Average of 2 microarray data of H2O2 treated cells was compared with 1 microarray data of control cells after normalization. For comparison with real time PCR, 12 RNA from 12 cell culture dishes in 2 groups obtained for microarray were used, and the result of real time PCR was expressed by average of GAPDH and $\Downarrow$-Actin. * p<0.05

Gene Symbol	Gene Name	Expression ratio by Microarray (H2O2/Controll)	Expression ratio by Real Time PCR(H2O2/Controll)
HMOX1	heme oxygenase (decycling) 1	4.73	2.06*
SOD1	superoxide dismutase 1, soluble	1.22	1.05
SOD2	superoxide dismutase 2, mitochondrial	1.17	1.36*
SOD3	superoxide dismutase 3, extracellular		1.89*
CAT	Catalase	0.84	1.08
GPX1	glutathione peroxidase 1	1.86	1.21*
GCLC	glutamate-cysteine ligase, catalytic subunit	2.28	2.27*
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	906.21	71.81*
JUNB	jun B proto-oncogene	7	3.65*
KEAP1	kelch-like ECH-associated protein 1	0.99	1.35
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F	15.59	9.18*
CYCS	cytochrome c, somatic	2.63	1.37*
CIDEA	cell death-inducing DFFA-like effector a	8.4	1.40*
OSGIN1	oxidative stress induced growth inhibitor 1	12.37	6.38*
REL	v-rel reticuloendotheliosis viral oncogene homolog	1.88	1.67*
NR4A3	nuclear receptor subfamily 4, group A members 3	136.75	24.9*
MAOA	monoamine oxidase A	2.6	2.16*
NFE2L2	nuclear factor (erythroid-derived 2)-like 2 (NRF2)	1.65	1.85*
NR4A1	nuclear receptor subfamily 4, group A, member 1	7.79	6.02*
CXCL3	chemokine (C-X-C motif) ligand 3	14.46	10.4*
GPR68	G protein-coupled receptor 68	6.42	2.08*
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	4.35	3.02*
TP63	tumor protein p63	34.72	1.86*
SEPP1	selenoprotein P, plasma, 1	10.55	2.46*
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	10.11	5.44*
BTK	Bruton agammaglobulinemia tyrosine kinase	9.51	4.35*
IL8	interleukin 8	7.51	3.70*
EGR1	early growth response 1	79.85	25.96*
NQO1	NAD(P)H dehydrogenase, quinone 1	0.86	1.39*

1   were treated with 100 µM H_2O_2 for 4 hours, then total RNA was extracted and labeled with Cy-

2   3 then analyzed by Agilent human whole genome microarray. Results were submitted to NCBI

3   GEO data base with accession number GSE83369.

4   (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE83369). Total 2903 genes were up-

5   regulated more than two times of untreated cells, and 2283 genes were down regulated less than

6   1/2. Most of the gene were classified unknown or others, the genes classified to nucleotide

7   metabolism were major group (Table 1, Supplemental Fig. S1, S2). Some of gene expression

8   was confirmed by RT- real time PCR (Table 1). Antioxidant enzyme *HMOX1*, was up-regulated,

9   but *SOD1* and *CAT* were not changed. On the other hand, *GPX1* and *GCLC*, a glutathione

10   synthesis and antioxidant enzymes, were increased. Among the transcription factors that are up-

11   regulated by H_2O_2 described by Marinho *et al.* (Marinho et al. 2014), *FOSB* and *JUNB* were

12   strongly up-regulated, suggesting AP-1 pathway was up-regulated. Several apoptosis related

13   enzymes were also up-regulated. Among the antioxidant pathway (Marinho et al. 2014), *KEAP1*

14   was not changed. There were many genes that function was unknown or slightly known, up-

15   regulated or down-regulated. Among them, *NR4A3*, kind of orphan receptor, was up-regulated

16   significantly, therefore, the effect of H_2O_2 concentration on the expression of this gene was

17   measured (Fig. 2A). The expression of *NR4A3* was increased by H_2O_2 concentration until 100

18   µM at 4 hours significantly. Therefore, the expression of *NR4A3* after H_2O_2 treatment was

19   measured up to 24 hours (Fig. 2B). It is interesting that *NR4A3* expression was increased rapidly

20   and decreased to original level by 24 hours, suggesting that the *NR4A3* is early responsible gene

21   to H_2O_2 stress, and it is suggested that NR4A3 might have important role in antioxidant defense

22   system in 1.1B4 cell against H_2O_2 stress.

23

24   **Knock down of *NR4A3* mRNA by RNA interference**

25   To investigate the roll of *NR4A3* in 1.1B4 cell, *NR4A3* mRNA was knocked down with siRNA.

26   The 1.1B4 cells were incubated with 5 nM siRNA of *NR4A3* for 48 hours, the mRNA of *NR4A3*

27   was decreased to 29.6% of control cell (Fig. 3A). The 1.1B4 cells knocked down with siRNA of

28   *NR4A3* were then treated with 100 µM H_2O_2, and analyzed cell viability by MTT assay (Fig.

29   3B). After 72 hours, the viability of siNR4A3 treated 1.1B4 cell was significantly decreased,

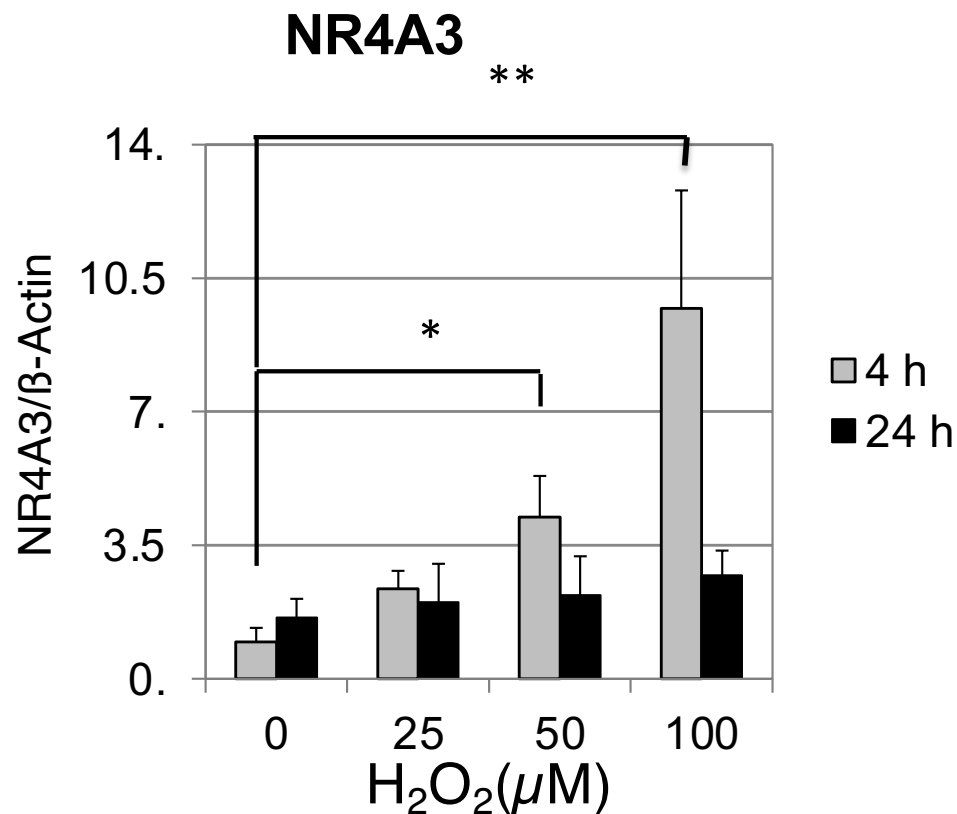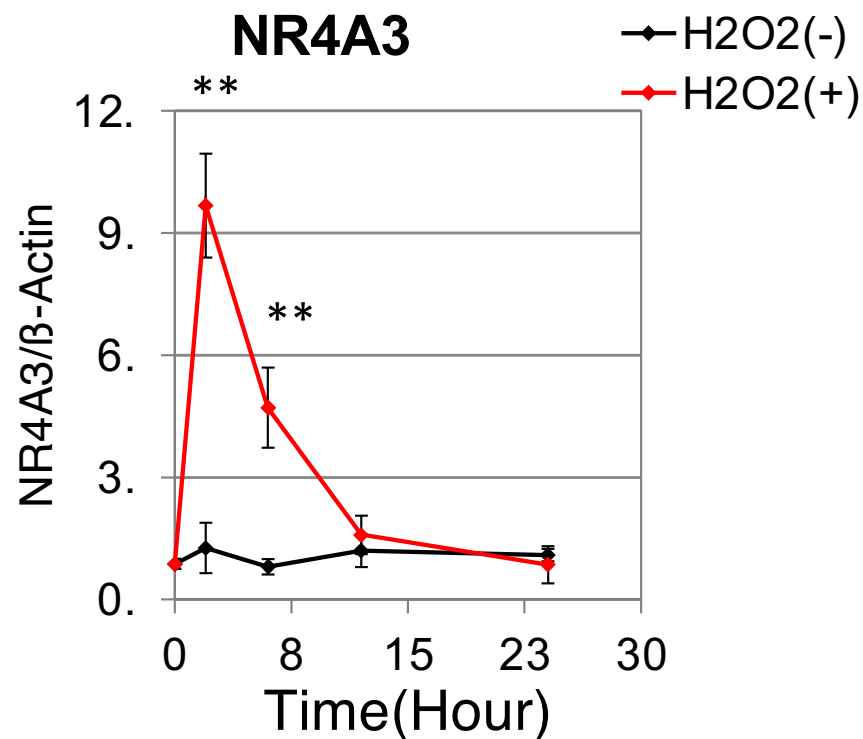30   and when H_2O_2 was added to culture medium, the growth was completely stopped.

Fig. 2. Effect of H_2O_2 concentration on *NR4A3* gene expression and time course of expression.
 A, Human 1.1B4 cell (1.0 x 10^5 cells/ml) was incubated in 2ml culture medium and 24 hours later medium was changed to individual concentration of H_2O_2 containing medium, then 4 or 24 hours later total RNA was extracted with Isogen. Expression of *NR4A3* was measured by real time PCR with condition B. n=3. **B**, After addition of 100 μM of H_2O_2, cell was incubated individual time and RNA was extracted. n=3. β-Actin was used for control.

Fig. 3.

B

NC   NFW   siNR4A3

Legend: H$_2$O$_2$(-) ; H$_2$O$_2$(+)
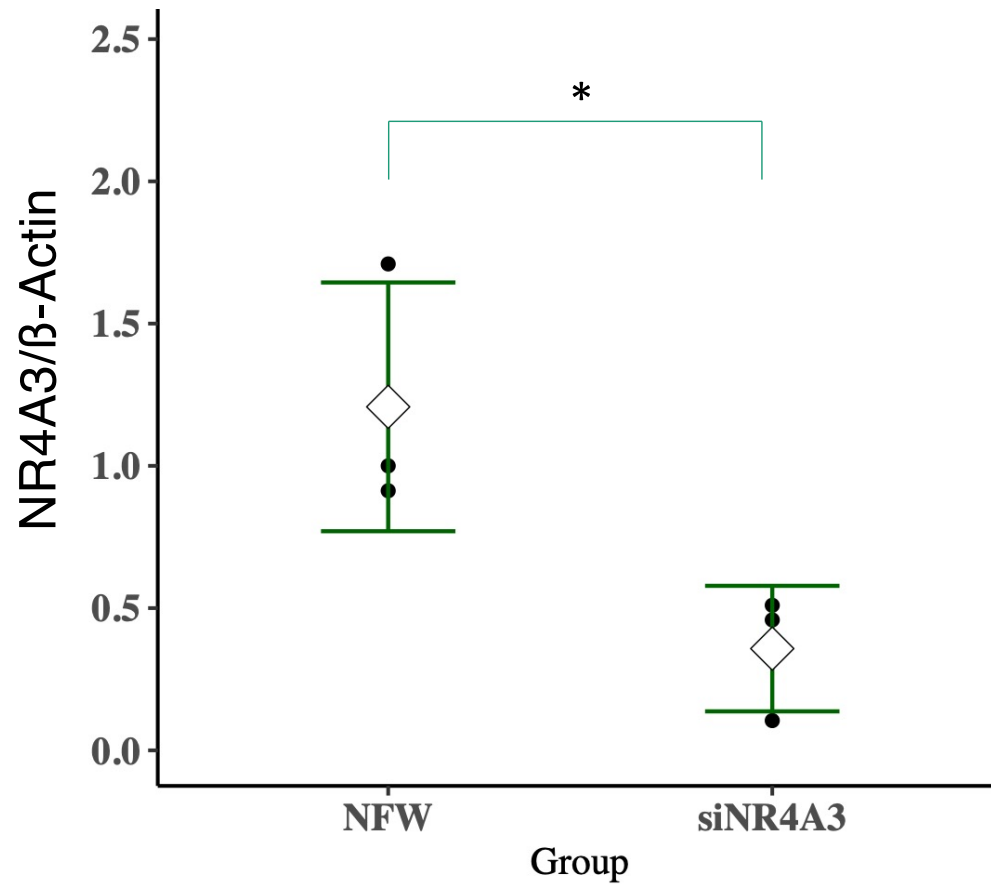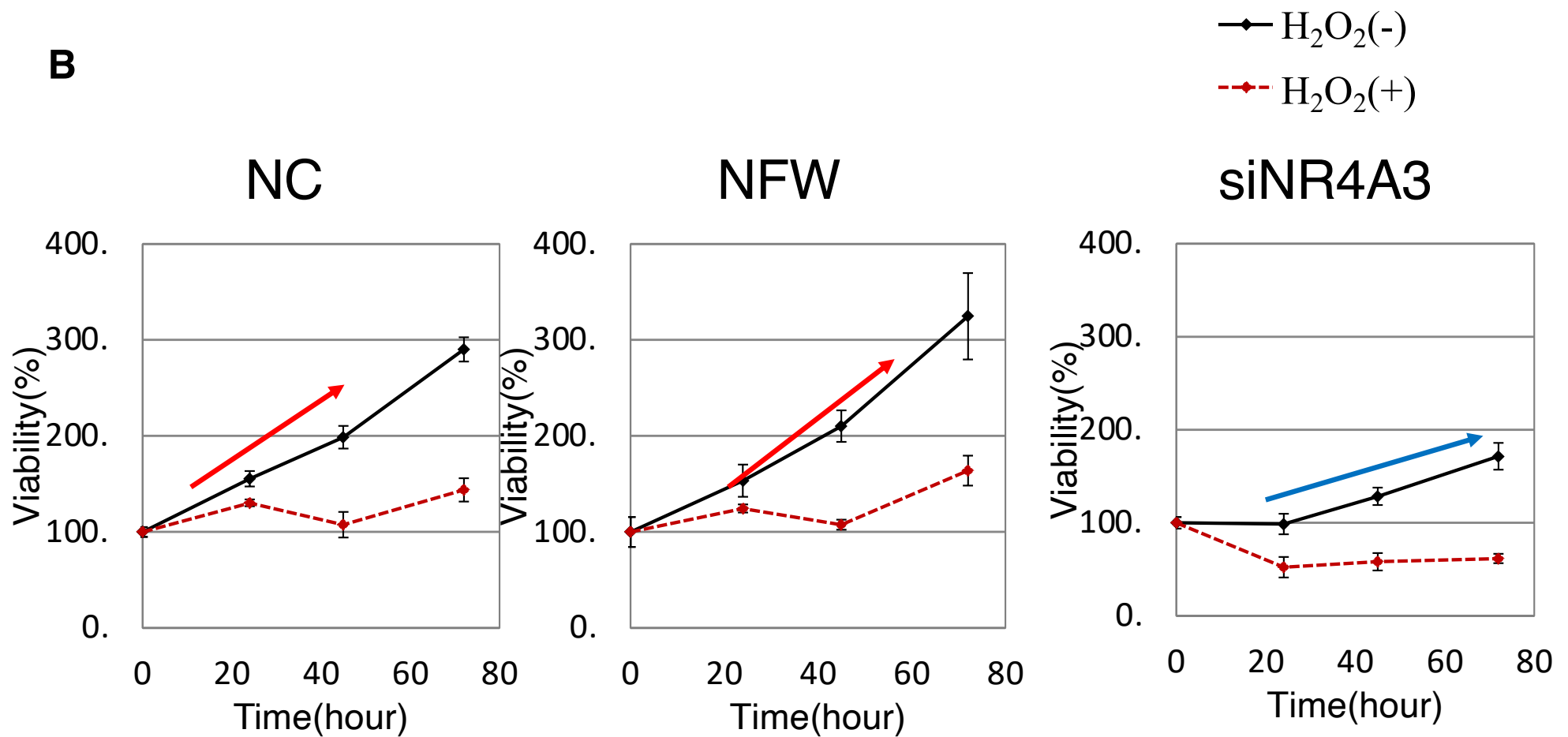
Fig. 3. Effect of *NR4A3* knocked down on the expression of *NR4A3* and cell viability. **A**, After knocked down of *NR4A3* gene in 1.1B4 cell with siRNA, total RNA was extracted and expression of *NR4A3* and β-actin was measured by real time PCR with Cyber green method with condition B. n=3 *p<0.05. **B**, After knocked down of *NR4A3* gene of 1.1B4 cell, cell was incubated with 100 μM of H$_2$O$_2$, and cell viability was measured by MTT assay. n=5. NC: negative control; NFW: nuclease free water added cell; siNR4A3: siRNA of *NR4A3* treated cell.

1

Comprehensive analysis of gene expression in 1.1B4 cells by DNA microarray after the

knock down of *NR4A3* with siRNA

To analyze the role of *NR4A3* in pancreatic islet cell further, we performed comprehensive

analysis of gene expression of 1.1B4 cells by Agilent human DNA microarray after the knocked

down of *NR4A3* mRNA. Data were submitted to NCBI GEO data base with accession number

GSE86924. (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE86924). We found 1,044

genes were significantly increased over 1.5 folds and 859 genes were significantly decreased

under 0.67 folds (Supplemental Fig. S3A, B). Using those genes, pathway analysis was

performed by KEGG pathway database. (Supplemental Table S2). Many genes in PI3K-Akt

signaling pathway were up-regulated, and Rap1 signaling pathway genes were down-regulated.

Changes in gene expression measured by microarray of transcription factor, related to H_2O_2

addition (Marinho et al. 2014), antioxidant genes and major cell growth related genes are also

listed in Table 2. Some of these results were confirmed by real time PCR (Fig. 4A - N,

Supplemental Fig. S4A - H). *NR4A3* expression was down regulated (Fig. 4A) but this group of

orphan receptor, *NR4A1* (Fig. 4B) was not changed and *NR4A2* (Fig. 4C) was up-regulated.

Among the genes of antioxidant enzymes, the expression of *HMOX1* was decreased when

measured by microarray, and confirmed by real time PCR (Fig. 4E), the expression of *HMOX1*

was reduced to 68%. However, *GCLC* (Supplemental Fig. S4E) was only 0.88 times of control,

and *SOD1* (Supplemental Fig. S4F) was not changed significantly. We found interesting gene

expression of antioxidant enzyme of *GLRX* (Fig. 4F). After the knocked down the *NR4A3*,

GLRX expression was increased, different from *HMOX1*. *SOD3* was also increased by *NR4A3*

knock down (Fig.4G). Among the transcription factors related to H_2O_2 oxidation, *MAFA* was

slightly down regulated measured by microarray but was not changed measured by real time

PCR (Supplemental Fig. S4A - D). Other redox sensitive transcription factors were not down

regulated.

Gizard *et al.* (Gizard et al. 2011) reported that NOR1 (NR4A3 or Nur77) is recruited to a nerve

growth factor-induced clone B response element (NBRE, aaaggaca or aaaggtca) site (Philips et

al. 1997). Therefore, whether the *HOMX1* gene has NBRE sequence, or not, was investigated.

The *HMOX1* gene structure was obtained from NCBI database (AY460337). There are two

NBRE like sequence of aaggtca, tentative NR4A3 responsible element, at second intron (Fig. 5).

Transcription factor responsible element in second intron might works when H_2O_2 is present in

1.1B4 cell culture medium.

Table 2. Changes of gene expression after siRNA of NR4A3 treatment measured by microarray.

Average of 3 microarray data of siRNA of NR4A3 treated cells after normalization was compared with average of 3 microarray data of control cells.

Gene symbol	Gene name	Fold changes (SiRNA vs Cont)	Gene symbol	Gene name	Fold changes (SiRNA vs Cont)
Orphan receptor			**Cell cycle**		
NR4A3	nuclear receptor subfamily 4, group A members 3	0.85	E2F1	E2F transcription factor 2	0.66688
NR4A2	nuclear receptor subfamily 4, group A, member 2	1.06	CDK1	cyclin-dependent kinase 1	0.7342
			CDK2	cyclin-dependent kinase 2	0.34304
Antioxidant responsibleTranscription Factor			CDK4	cyclin-dependent kinase 4	0.87803
MAFA	v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (avian)	0.75	CCNA1	cyclin A1	0.84123
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	0.96	CCNB1	cyclin B1	0.72389
FOS	FBJ murine osteosarcoma viral oncogene homolog	2.5	CCND1	cyclin D1	0.99547
JUNB	jun B proto-oncogene	1.02	CCNE1	cyclin E1	0.44222
JUN	jun proto-oncogene	0.76	CCNY	cyclin Y	0.62658
KEAP1	kelch-like ECH-associated protein 1	0.93	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	1.66505
NFE2L2	nuclear factor (erythroid-derived 2)-like 2, NRF2	No data	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	1.66505
CREB5	cAMP responsive element binding protein 5	2			
TP53	tumor protein p53	0.83	**Others**		
NOTCH4	notch 4	1.36	RAB5B	member RAS oncogene family (RAB5B)	0.54527
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.95	RAP1B	RAP1B, member of RAS oncogene family	0.58507
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	0.77	TGFB3	transforming growth factor, beta 3	0.50684
SP1	Sp1 transcription factor	0.8	PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide	1.24095
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	1.02	MRAS	muscle RAS oncogene homolog	0.4872
SREBF1	sterol regulatory element binding transcription factor 1	0.76	RAP1B	RAP1B, member of RAS oncogene family	0.59426
HSF1	heat shock transcription factor 1	0.84	SREBF1	sterol regulatory element binding transcription factor 1 (A_33_P3222139)	0.85885
			HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	0.91634
Antioxidant Responsible Enzyme			BAD	BCL2-associated agonist of cell death	0.65799
HMOX1	heme oxygenase (decycling) 1	0.48585	AKT1	v-akt murine thymoma viral oncogene homolog 1	0.85096
GCLC	glutamate-cysteine ligase, catalytic subunit	0.88148	BAD	BCL2-associated agonist of cell death	2.09416
GPX1	glutathione peroxidase 1(A_33_P3239849)	0.81867	FOXO1	Homo sapiens forkhead box O1 (FOXO1), mRNA [NM_002015]	1.0298
GPX1	glutathione peroxidase 1(A_33_P3354322)	0.81248	FOXO3	Homo sapiens forkhead box O3 (FOXO3), transcript variant 1, mRNA	1.08933
CAT	catalase	1.59628	GAPDH	glyceraldehyde-3-phosphate dehydrogenase	0.73695
SOD1	superoxide dismutase 1, soluble	0.87111	TBP	TATA box binding protein	0.93576
SOD2	superoxide dismutase 2, mitochondrial	0.6454	ACTB	actin, beta	0.89854
SOD3	superoxide dismutase 3, extracellular	0.65966			
GLRX	glutaredoxin (thioltransferase)	1.97201			
GLRX2	glutaredoxin 2	0.83005			
TXN	thioredoxin	0.82374			
PMEL	premelanosome protein	1.6221			
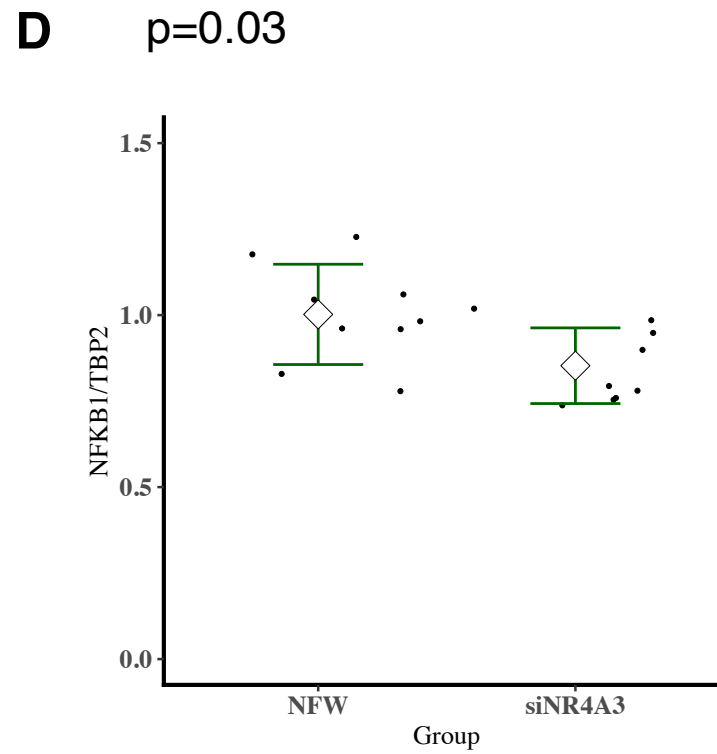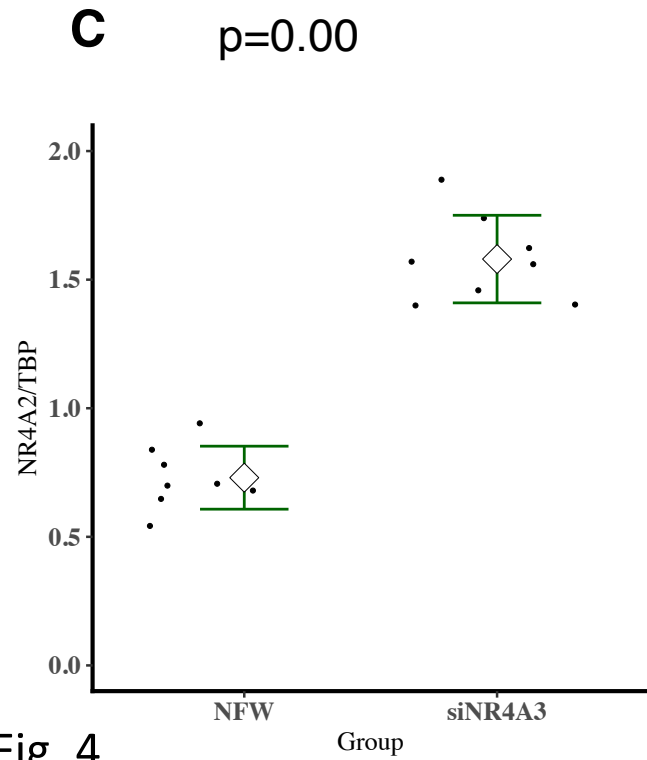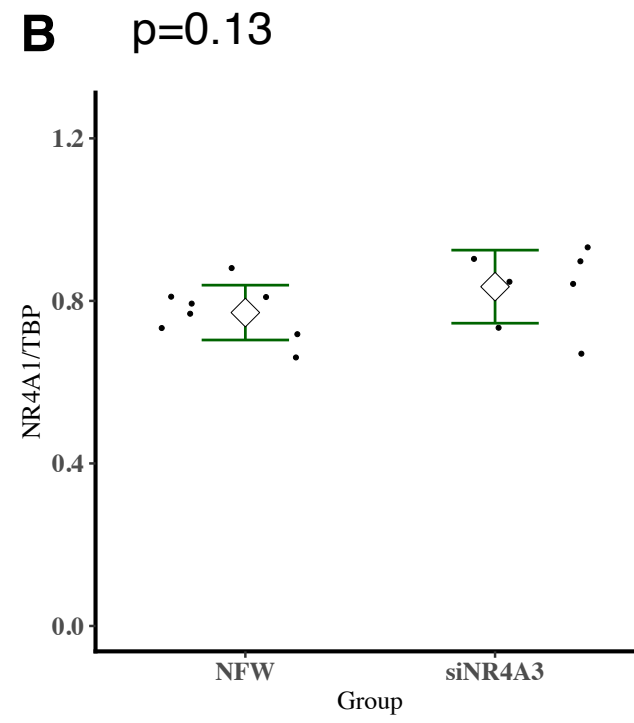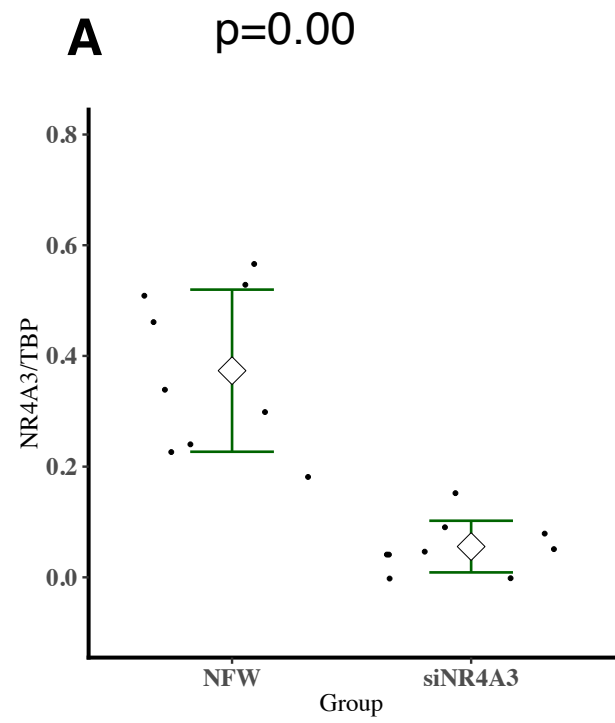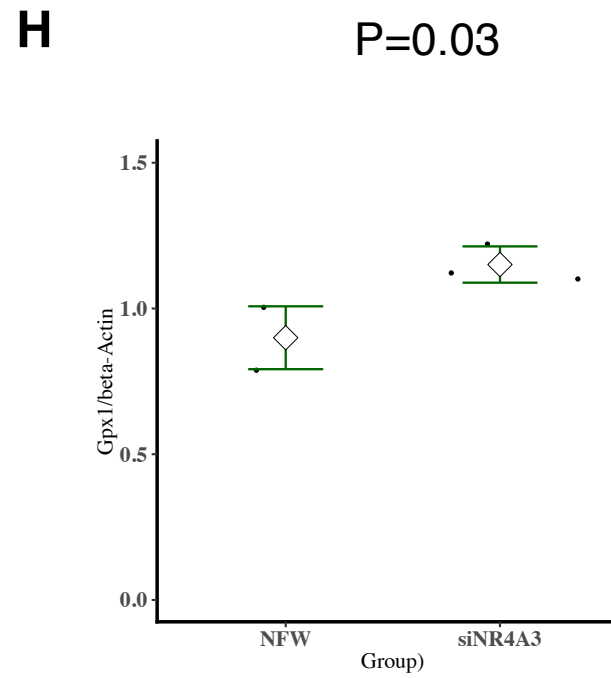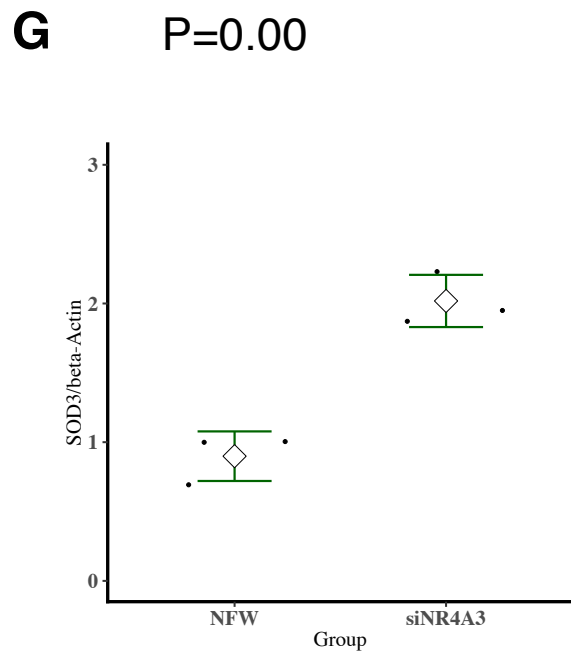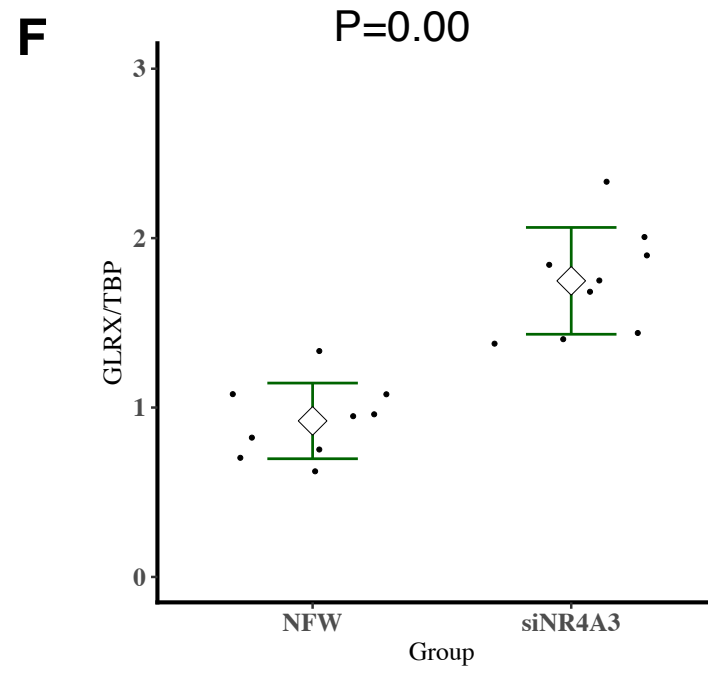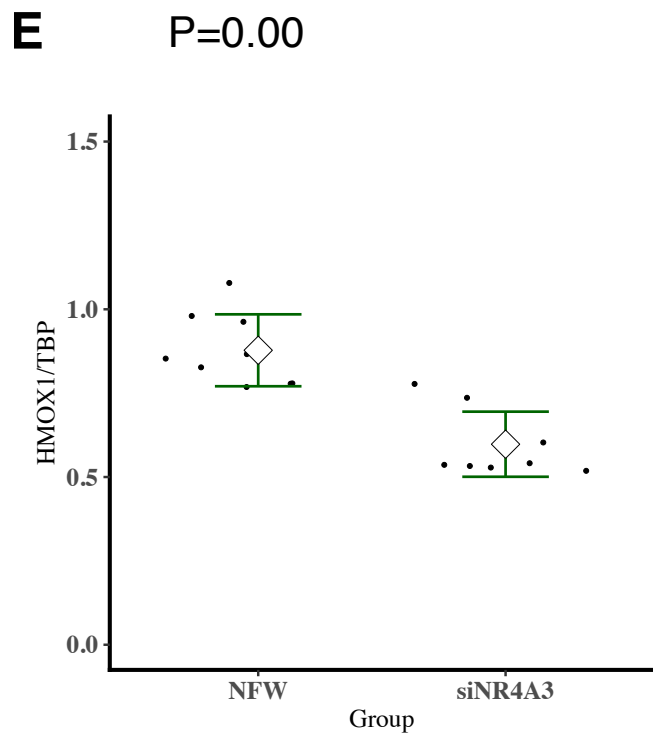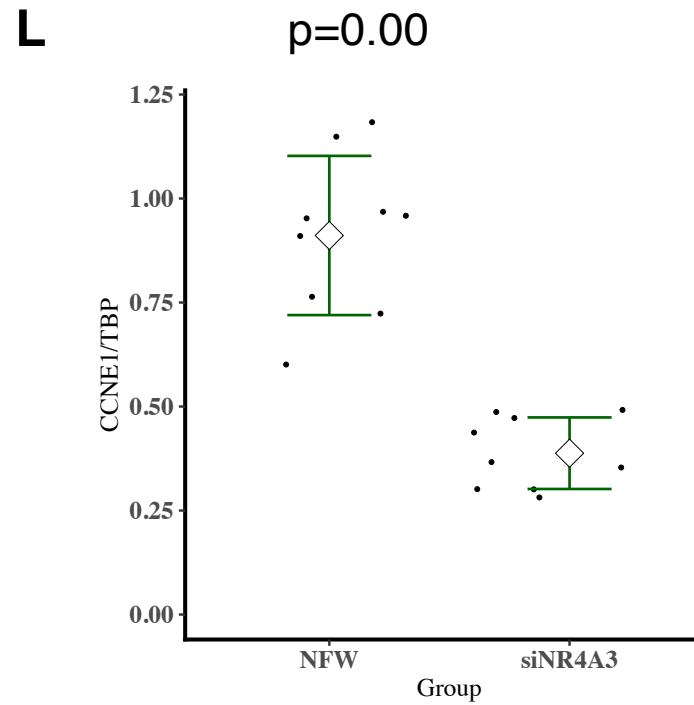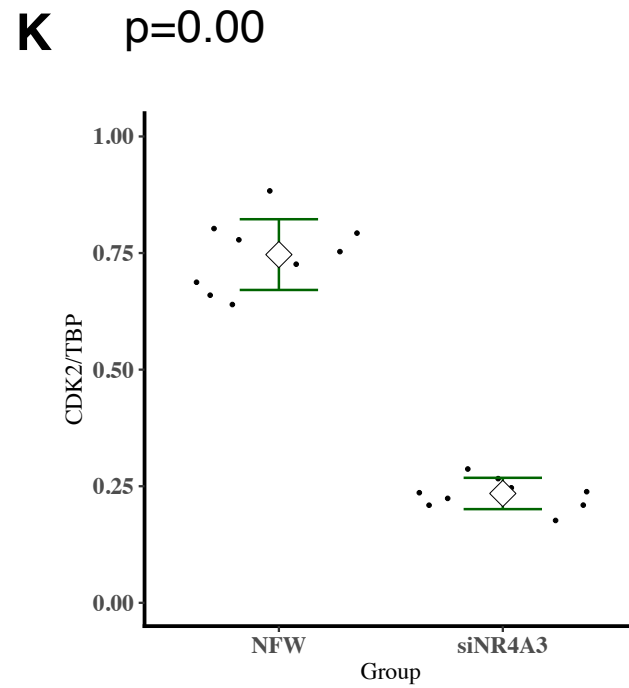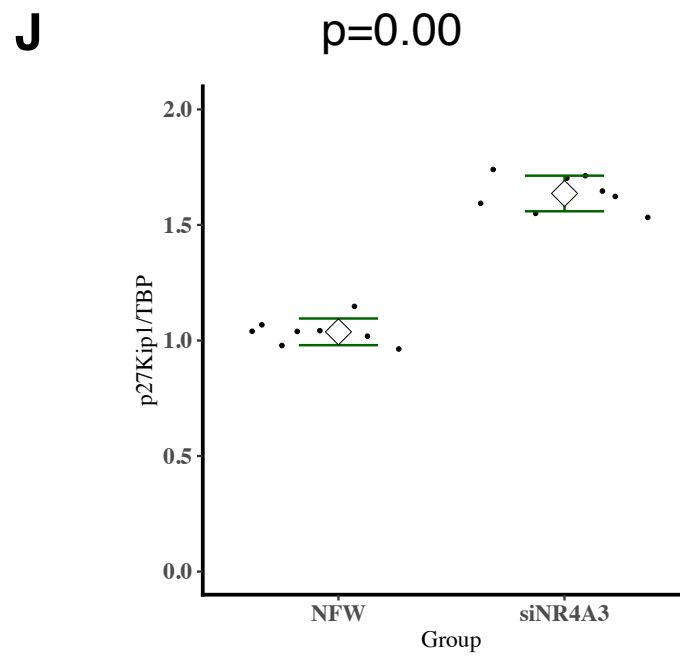
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Fig. 4.

Fig. 4.

Fig. 4.

Fig. 4. Changes of Various Gene expression in 1.1B4 Cell after the knocked down of *NR4A3* mRNA measured by real time PCR.
(A) Human 1.1B4 cells were incubated 24hours, half of them (n=9) were added siRNA (siNR4A3) and another half (n=9) were added nuclease free water (NFW), and after 52 hours, mRNA was purified with RNeasy Plus Mini Kit. Expression of *NR4A3* was measured by real time PCR with Taqman method using condition C. TATA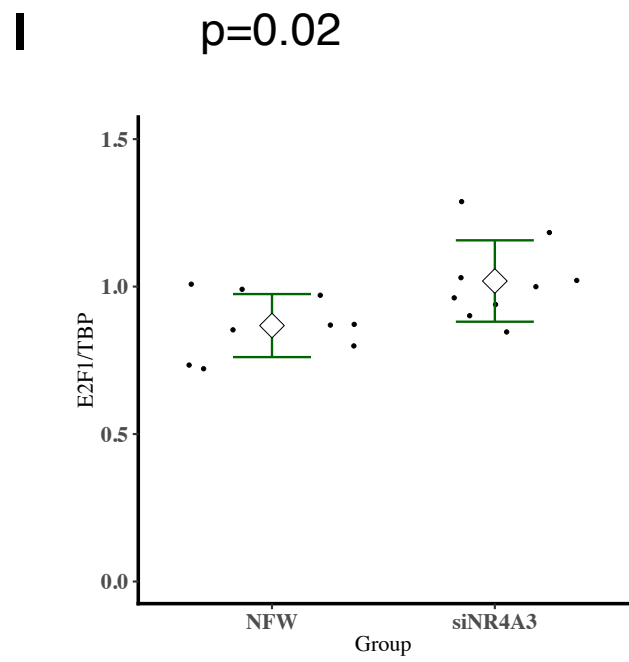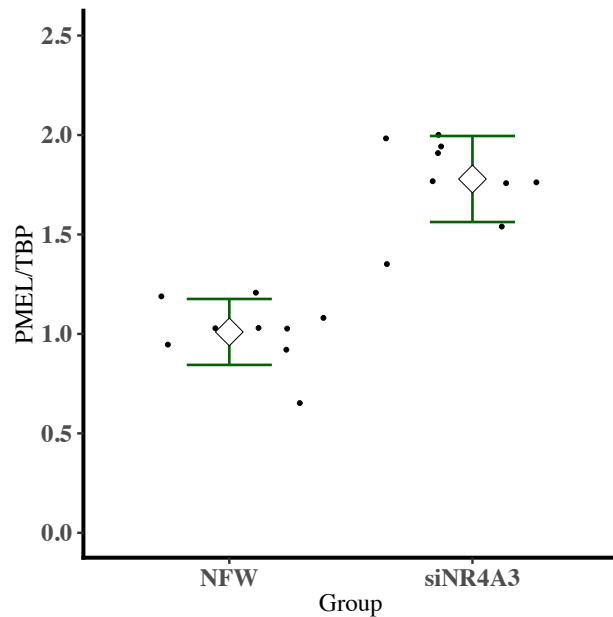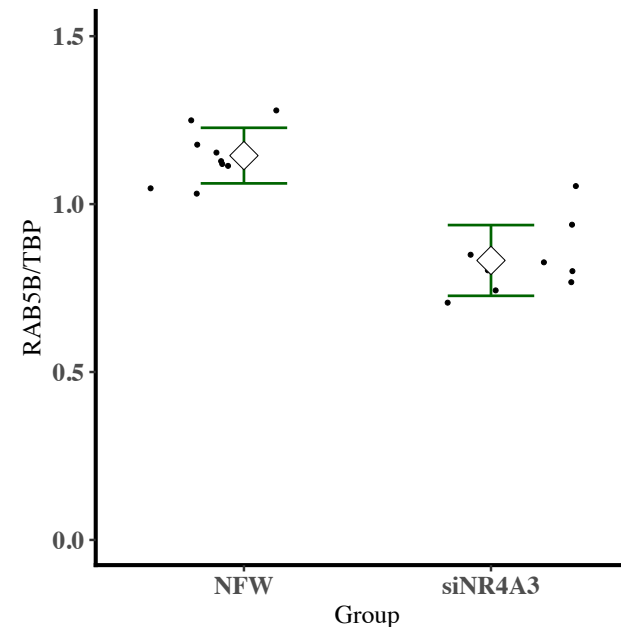 binding protein (TBP) mRNA was used for internal control. (B) The *NR4A1* expression, (C) *NR4A2* expression. (D) - (E) *NFKB1*and *HMOX1* respectively. (F) *GLRX* expression (n=8). (G) Human 1.1B4 cells were incubated 24hours, half of them (n=3) were added siRNA (siNR4A3) and another half (n=3) were added NFW, and after 52 hours, mRNA was purified with Isogen reagent and treated with RNase-free DNase. Expression of *SOD3* was measured by real time PCR with Cyber green method using condition B. b-Actin was used for control. (H) Expression of *GPX1* using same method of (G). (I) *E2F1* using same method of (A) (n=8). (J) - (M) *p27Kip1, CDK2, CCNE1* and *PMEL*, respectively, using same method of (A). (N) *RAB5B* using same method of (A) (n=8).
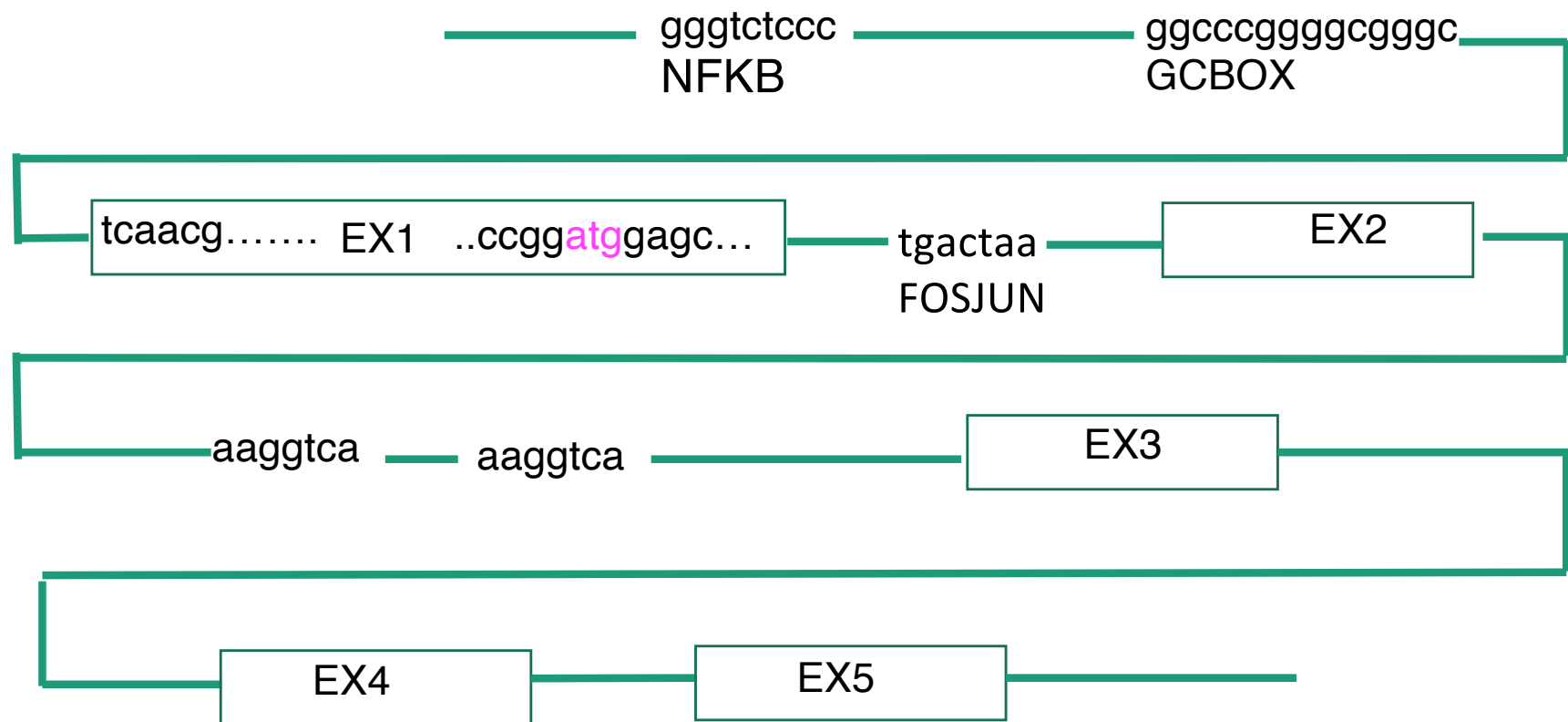
Fig. 5. Gene structure of *HMOX1*.
 Human *HMOX1* gene was obtained from PubMed gene (ACCESSION AY460337). NR4A3 binding motif of aaggtca were searched and abstract of the gene structure was displayed. NFkB shows NFkB binding motif. FOSJUN shows FOS and JUN binding motif. EX1 *etc*. shows exson 1 *etc*.

As described before, knock down cells decreased cells growth, therefore, cell cycle regulation genes that expression was modified by siRNA of *NR4A3*, were investigated. *CCNE1* (*Cyclin E*) and *CDK2* expressions were decreased remarkably (Table 2). Then those down-regulations were confirmed by RT-real time PCR (Fig. 4K, L). *CDK2* and *CCNE1* expression were decreased strongly compared with un-treated cell. To investigate the mechanism, gene structure of *CDK2* and *CCNE1* were analyzed (Supplemental Fig. S5A, B and S6). There was no aaggtca at up-stream of *CDK*2, however, down-stream of *CDK2*, there is aaggtca sequence. This area is also up-stream of the gene of *RAB5B*, therefore, the effect of siRNA of *NR4A3* on this *RAB5B* expression was investigated. There is also *PMEL* is near this area, the expression of *PMEL* was also measured. As shown in Fig 4M and N, those two genes expression were also decreased or increased by siRNA of *NR4A3*. It is, therefore, suggested that *NR4A3* promotes proliferation through activating *Rap1* signaling pathway, therefore, it is also suggested that the *NR4A3* contribute to homeostasis against extracellular stress such as oxidative stress.

DISCUSSION

It is known that pancreatic islet ß-cell weakly express antioxidant enzymes, therefore, oxidative stress enhances diabetes mellitus. As shown in this experiment of microarray of H_2O_2 treated 1.1B4 cell, among the antioxidant enzymes, *HMOX1, GPX1* and *GCLC* were up-regulated (Table 1). Other major antioxidant enzymes were not up-regulated, for example, *SOD1* and *CAT* were not induced by H_2O_2 addition, therefore, those *HMOX1, GPX1* and *GCLC* enzymes are responsible for antioxidant defense system in pancreatic cell, and those are very important enzymes for defending against oxidative stress at diabetes. Alam and Cook (Alam and Cook 2007) described that there are 4 pathways that regulates *HMOX1* gene expression at oxidative stress. But here, not only those 4 pathway, we showed that *HMOX1* and *NR4A3* were up-regulated when H_2O_2 was added, and the expression of *HMOX1* was down-regulated when *NR4A3* was knocked down by siRNA resulting loss of antioxidative resisstance. Furthermore, the second intron of *HMOX1* has NBRE like sequence of aaggtca. Therefore, we concluded that the *NR4A3* is antioxidant responsible transcription factor and regulates *HMOX1* expression at oxidative stress. Not only 1.1B4 cell, *NR4A3* expression was also increased in HUCF2 cell at oxidative stress (Shimizu et al. 2015). Therefore, we propose that the *NR4A3* is new oxidative stress responsible transcription factor not listed before (Marinho et al. 2014), and this pathway is major antioxidant pathway in 1.1B4 cell (Graphical abstract). *FOSB* is also up-regulated by

Graphical abstract
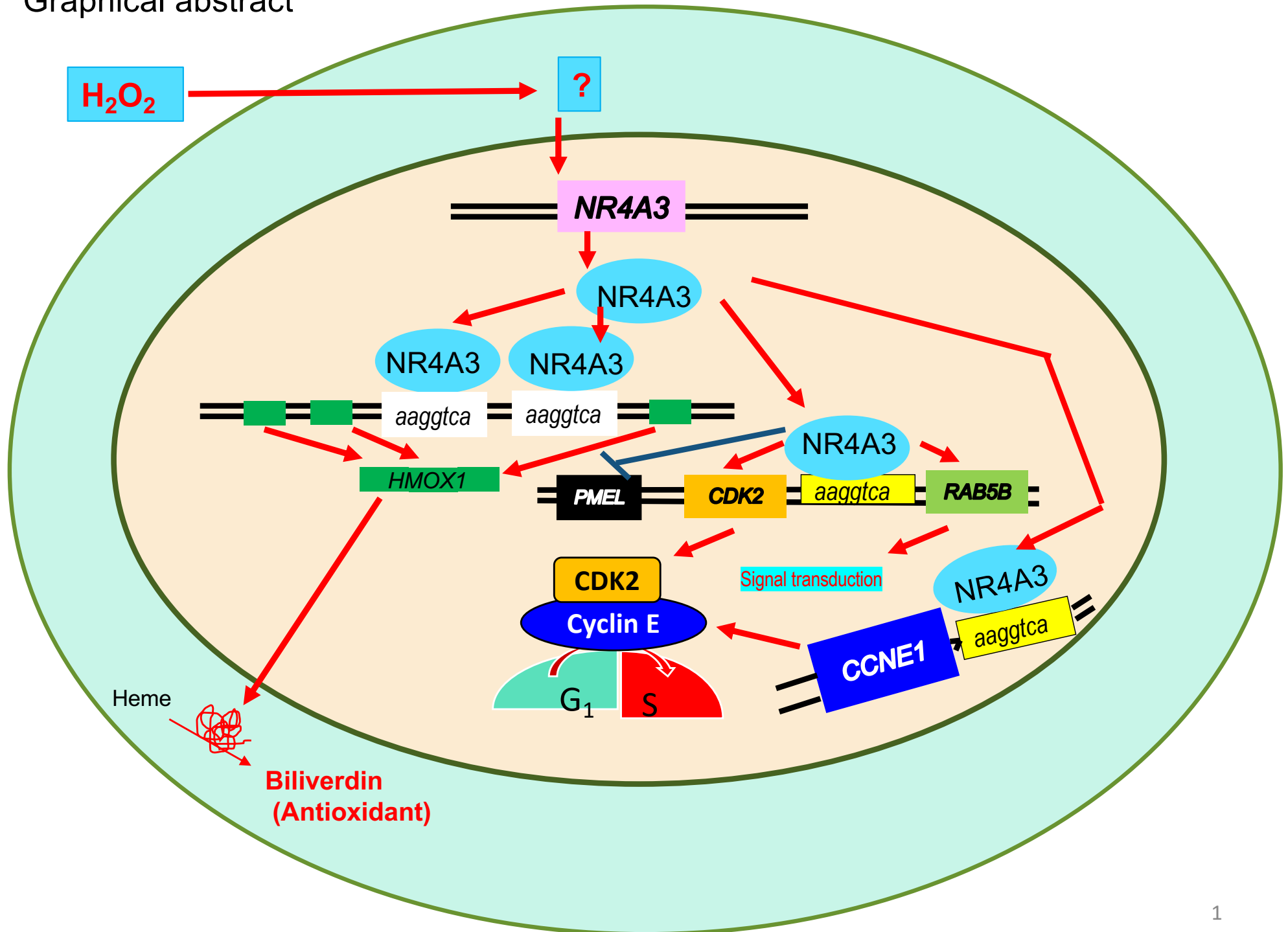
Hydrogen peroxide induces NR4A3 and binds to aaggtca sequence of HMOX1, and increased transcription of HMOX1. Resulting heme oxygenase produces biliverdin, antioxidants, from heme. NR4A3 also bind to aaggtca sequence of CDK2 and CCNE1, resulting CDK2 and Cyclin E. CDK2 bind to cyclin E and cell goes from G1 to S phase.

1   H_2O_2 in this experiment, therefore, FOS pathway might be also another major antioxidant

2   pathway.

3   There are two NBRE like sequence of aaggtca in *HMOX1*. NBRE contains aggtca which is

4   typically recognized by retinoic acid receptor/retinoid X receptor (RAR/RXR) subfamily, and it

5   also includes two aa residues preceding this hexanucleotide (Philips et al. 1997). On the other

6   hand, NR4A1 and NR4A2 forms heterodimer with RXR and binds to direct repeat of aggtca

7   (Safe et al. 2016). Therefore, we propose aaggtca is NR4A3 responsible element.

8

9   Micro-array analysis has shown that knock down of *NR4A3* decreased *CDK2* and *CCNE1*

10   (*cyclin E*) expression (Table 2). This result confirmed by real time PCR and promotor region

11   analysis (Fig. 4K and 4L, Supplemental Fig. S5A, B and S6). These results show that *NR4A3* is

12   key transcription factor not only for antioxidant system but also for cell cycle control in 1.1B4

13   cell. Tessem *et al.* (Tessem et al. 2014) demonstrate that *E2F1* and *CCNE1* are key cell cycle

14   inducers. Our result of *cyclin E* consistent with Tessem results, but our results showed *E2F1*

15   expression was only slightly increased in *NR4A3* knock down cell (Fig. 4I). Therefore, it is

16   suggested that NR4A3 directly controls *cyclin E* and *CDK2,* not via *E2F1*.

17

18   Robertson (Robertson 2010) described that main stays of therapy for type 2 diabetes involve

19   drugs that are insulin-centric, i.e., they are designed to increase insulin secretion and decrease

20   insulin resistance. The mechanism for this unrelenting deterioration of ß-cell function is related

21   to chronic oxidative stress. This suggests that drug discovery should not exclusively focus on

22   insulin-centric targets, but also include glucose-centric strategies, such as antioxidant protection

23   of the ß-cells. This may facilitate repair of ß-cells undergoing damage by oxidative stress

24   secondary to chronic hyperglycemia. Furthermore, Gao *et al*. (Gao el al. 2014) have shown that

25   over expression of *NR4A3* results in down-regulation of insulin gene transcription and insulin

26   secretion. It is suggested that oxidative stress up-regulated *NR4A3* expression resulting down-

27   regulation of insulin secretion. Therefore, antioxidant material might recover from islet damage.

28   For this aim, many antioxidants were used for diabetes therapy. For example, Cinnamtannin D-

29   1, one of the main A-type procyanidin oligomers in *C. tamala*, was discovered to dose-

30   dependently reduce palmitic acid- or H_2O_2-induced apoptosis and oxidative stress in INS-1 cells,

31   MIN6 cells, and primary cultured murine islets (Wang et al. 2014), however, antioxidant drugs

32   were not in use for human diabetes. Therefore, we screened new antioxidant from food stuff for

1 potential therapy use, and recently found strong antioxidant Zeylaniin A from edible vegetable

2 (Nomi et al. 2012). This polyphenol may be used for diabetes treatment.

3

4 For microarray experiment, Agilent array was used and confirmed by real time PCR. Some

5 times other type of microarray shows different data from real time PCR and shows false positive

6 data, but Agilent array used here shows very co-related data to real time PCR as far as we

7 measured in this experiment. And three microarray data of same conditions showed very similar

8 data. Use of internal control gene for real time PCR was not easy, because many house keeping

9 genes are known to change their transcription depend on the conditions. Therefore, average of

10 two house keeping genes of *GAPDH* and *β-actin* were used for H_2O_2 experiment. *TBP* was also

11 used for siRNA treated cell because *TBP* was not changed by siNR4A3 treatment measured by

12 microarray (Table 2).

13

14 **METHODS**

15 **Reagents**

16 RPMI-1640 medium (GIBCO, Tokyo or Sigma, Tokyo), FBS (CELLect FBS, MP Bomedical

17 Japan, Tokyo Japan, LOT#7997K or Biowest, Nuaillé, France, Lot.No:S05831S1820), Penicilin

18 (50 IU/ml) - Streptomycine (50 micro g/ml) (ICN Biomedicals, Irvine, California, United

19 States), 96 well plate (IWAKI, Tokyo, Japan), NR4A3 Silencer Select siRNA (Applied

20 Biosystems - Thermo Fisher Scientific Japan, Tokyo, siRNA ID : s15542), Silencer Select

21 Negative Control #1 siRNA, Nuclease Free Water and Lipofectamine 2000 (Applied

22 Biosystems - Thermo Fisher Scientific Japan, Tokyo). RNeasy Plus Mini Kit (Qiagen, Tokyo,

23 Japan), Bio-analyzer (Agilent Technology, Palo Alto, CA, USA). Isogen reagent and RNase-

24 free DNase (Nippon gene, Toyama, Japan). Agilent Whole Human Genome Oligo Microarray

25 4x44K Ver. 2.0 (Agilent Technology, Palo Alto, CA, USA) Reagents for microarray (Agilent

26 Technology, Palo Alto, CA, USA). Agilent G2565BA Microarray Scanner System (Agilent

27 Technology, Palo Alto, CA, USA). The scanned images were analyzed with Feature Extraction

28 Software 9.5.1.1 (Agilent Technology, Palo Alto, CA, USA) using default parameters (protocol

29 GE1-v5_95_feb07 and Grid: 014850_D_F_20101031). Spot Fire software (TIBCO, NTTCom,

30 Tokyo, Japan) and the GeneSpringGX10 v 7.3.1 (Agilent Technology, Palo Alto, CA, USA).

31 DNaseI-treated total RNA with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA)

1   and Oligo (dT) 15 primer (Promega, Madison, WI, USA). STEP ONE PLUS Real Time PCR

2   system (Applied Biosystems Japan, Tokyo, Japan), SYBR GREEN PCR Master Mix (Applied

3   Biosystems Japan, Tokyo, Japan). Takara Premix Ex Taq (Probe qPCR) (TaKaRa, Kyoto,

4   Japan), Designs of PCR probes: Universal Probe Library Assay Design Center at Roche

5   (http://www.universalprobelibrary.com). ABI PRISM 7900HT Sequence Detection System

6   (Applied Biosystems, Tokyo, Japan).

7   **Biological Resources: Human pancreatic islet derived cell, 1.1B4**

8   Islet derived hybrid cell of 1.1B4 (ECACC No. 10012801) formed by the electrofusion of a

9   primary culture of human pancreatic islets with PANC-1, a human pancreatic ductal carcinoma

10   cell line (ECACC catalogue number 87092802), was obtained from DS Pharma Biomedical Co.,

11   Ltd., Osaka, Japan, and cultured in RPMI-1640 medium (GIBCO, Tokyo or Sigma, Tokyo,

12   Japan) supplemented with 10% FBS (CELLect FBS, MP Bomedical Japan, Tokyo Japan,

13   LOT#7997K or Biowest, Nuaillé, France, Lot.No:S05831S1820) with Penicilin (50 IU/ml) -

14   Streptomycine (50 μg/ml) (ICN Biomedicals, Irvine, CA, USA) in humidified air at 37 °C with

15   5% CO_2.

16

17   **Cell toxicity of H_2O_2 and the effect of H_2O_2 on gene expression of 1.1B4**

18   The cells of 1.1B4 were cultured in 96 well plate (IWAKI, Tokyo, Japan) at 6.0 x 10^3

19   cells / well with RPMI-1640 medium for 24 hours, then 0, 10, 20, 30, 40, 50, 100, 200

20   μM H_2O_2 was added to medium, and cell viability was analyzed at 24, 48 and 72 hours

21   by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric

22   assay (Mosmann 1983). We found that there was no toxicity up to 150 μM H_2O_2. Next,

23   we examined the effect of H_2O_2 on gene expression by real time PCR. Human 1.1B4

24   cell (1.0 x 10^5 cells/ml) was incubated in 2ml culture medium and 24 hours later

25   medium was changed to 25, 50 and 100 μM H_2O_2 containing medium, then 4 or 24

26   hours later total RNA was extracted with Isogen. Expression was measured by real time

27   PCR with condition B. Cells of 1.1B4 were also treated with 100 μM H_2O_2 and we

28   analyzed gene expression after the exposure to H_2O_2 for 2, 6, 12, 24 hours.

29

1   **Knock down of NR4A3 mRNA by RNA interference**

2   NR4A3 Silencer Select siRNA (ABI, siRNA ID: s15542) was used for knocked down the

3   *NR4A3* mRNA, and Silencer Select Negative Control #1 siRNA (ABI) and Nuclease Free Water

4   (ABI) were used for negative control. *NR4A3* mRNA of 1.1B4 cells was knocked down by

5   incubating with 5 nM siRNA and 0.1 % of Lipofectamine 2000 for 48 h. Total RNA was

6   isolated by Isogen then mRNA was measured as described before. The 1.1B4-siNR4A3 treated

7   cells or control cells were treated with or without 100 μM H_2O_2, and cell viability was assayed

8   by MTT assay.

9

10   **Extraction and purification of mRNA**

11   For micro-array, total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Tokyo,

12   Japan). The purity of RNA was assessed by a Bio-analyzer (Agilent Technology, Palo Alto, CA,

13   USA) before microarray analysis. The RNA thus obtained was also used for real time PCR. For

14   only real time PCR use, total RNA was extracted with Isogen reagent (Nippon gene, Toyama

15   Japan) as described previous paper (Oba et al. 2006) and treated with RNase-free DNase

16   (Nippon gene, Toyama, Japan).

17

18   **Global analysis of gene expression by DNA microarray in 1.1B4 cells**

19   **(Experiment A)** Pancreatic derived 1.1B4 cell was seeded as 1.5×10^5 cells / ml to RPMI-1640

20   medium (GIBCO, Tokyo, Japan) in 6 well plate, and 24 hours later, cell was incubated with 100

21   μM H_2O_2 4 hours, then total RNA was extracted with RNeasy Mini Kit (Qiagen, Tokyo) (n=6

22   for control and treated respectively). The quality of all 12 RNA samples was checked by Agilent

23   RNA 6000 Nano Reagents Part1 (Agilent Technologies, Tokyo, Japan), and 3 samples of RNA

24   were combined to one microarray sample, then analyzed by Agilent human microarray by the

25   protocol of manufacture. In brief, Cy3-labeled cRNA was generated from 200 ng input total

26   RNA using Agilent's Low Input Quick Amp Labeling Kit (Agilent Technologies, Tokyo,

27   Japan). cDNA was generated with the primer containing a T7 polymerase promoter, and then

28   was transcribed into cRNA in accompany with the dye labeling. For every type of cell, 1.65 μg

29   cRNA from each labeling reaction was hybridized to the Agilent Whole Human Genome Oligo

30   Microarray (Agilent Technologies, Tokyo, Japan). The Whole Human Genome Oligo

31   Microarray is in a 4 x 44k slide format and each block represents more than 41,000 unique

1   human genes and transcripts. After hybridization, the slides were washed and then scanned with

2   the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Tokyo, Japan). The

3   scanned images were analyzed with Feature Extraction Software 9.5.1.1 (Agilent Technologies,

4   Tokyo, Japan) using default parameters (protocol GE1-v5_95_feb07 and Grid:

5   014850_D_F_20101031) to obtain background subtracted and spatially detrended Processed

6   Signal intensities. Features flagged in Feature Extraction as Feature Non-uniform outliers were

7   excluded. Data were further normalized using Gene Spring GX10 using default parameters of

8   recommended protocol with Median shift normalization to the 75th percentile and baseline

9   transformed to the median of all samples. Data normalization and filtering was performed using

10   Spot Fire software (TIBCO, NTTCom, Tokyo, Japan) and the Gene Spring v 7.3.1 (Agilent

11   Technologies, Tokyo, Japan). After the reading, one of microarray for control sample was

12   scratched, therefore, the gene expression was compared average of two treated array samples vs

13   one control sample. These 12 RNA samples were also used for real time PCR analysis.

14   **(Experiment B)** The microarray experiment was designed as follows. Human 1.1B4 cells (n=24)

15   were incubated in RPMI-1640 medium 24hours, half of them (n=12) were added siRNA

16   (siNR4A3) and another half (n=12) were added nuclease free water (NFW), and 48 hours later 3

17   cells of each group were added H_2O_2 to 100 mM. Those 6 cells samples were stored but not

18   used for this paper. After 52 hours, mRNA of remaining 18 cells was purified with RNeasy Plus

19   Mini Kit. The quality of mRNA was checked by Bio-analyzer, 3 mRNA samples were

20   combined to one microarray sample (3 knocked down microarray samples and 3 control

21   microarray samples), and applied on microarray analysis with Agilent Whole Human Genome

22   Oligo Microarray 4 x 44k. The expression was analyzed by Gene-spring software and Spot fire

23   software. Average of 3 array samples treated by H_2O_2 was compared to average of 3 array

24   samples without treated by H_2O_2. For real time PCR, 18 RNA samples were used individually.

25   Real time PCR with Taqman method was used (condition C). *TBP* mRNA was used for internal

26   control. All the sample-labeling, hybridization, washing, and scanning steps were conducted

27   following the manufacturer's specifications as described before.

28

29   **Real-time RT-PCR**

30   The cDNAs were synthesized from DNaseI-treated total RNA with M-MLV reverse

31   transcriptase (Invitrogen, Carlsbad, CA, USA) and Oligo (dT) 15 primer (Promega, Madison,

32   WI, USA) according to the manufacturer's instructions. Analysis of gene expression was

1   performed with a STEP ONE PLUS Real Time PCR system (Applied Biosystems Japan, Tokyo,

2   Japan) using SYBR GREEN PCR Master Mix (Applied Biosystems Japan, Tokyo, Japan) and

3   the primers listed in Supplemental Table S1. **Condition A**, 0.2 µl of cDNA was mixed with 0.1

4   µl forward primer (50 pmol / ml), 0.1 µl reverse primer (50 pmol / ml), 4.7 µl water and 5 µl

5   2×SYBR Green PCR Master MIX. Reactive condition was first incubated at 50 °C for 2 min

6   and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 1 min, then final

7   of 95 °C for 15 sec, 60 °C for 15 sec and 95 °C for 15 sec. Normalization of the data was

8   achieved by quantitating the cycle time at an arbitrary fluorescence intensity in the linear

9   exponential phase using Step One Plus Real-Time system Software (Applied Biosystems Japan,

10 Tokyo, Japan) by calculating the ratio of the relative concentration of each mRNA relative to

11 that of average of *GAPDH* and *ß-actin* or *ß-actin* only. To confirm amplification specificity, the

12 PCR products from each primer pair were subjected to a melting curve analysis. The relative

13 quantification of gene expression was computed by using the comparative Ct (threshold cycle)

14 method. **Condition B**, 0.2 µl of cDNA was mixed with 1 µl forward primer (50 µM), 1 µl

15 reverse primer (50 µM), 2.8 µl water and 5 ml 2×SYBR Green PCR Master MIX with same

16 reaction condition.

17 **Condition C** was performed with a TaqMan method. Briefly, the synthesized cDNA products

18 were subjected to real-time PCR in a reaction mixture (10 µl) containing Takara Premix Ex Taq

19 (Probe qPCR) (TaKaRa, Kyoto, Japan), 200 nM hydrolysis probes and 200 nM PCR primers.

20 Two µl of cDNA was mixed with 5 µl of Premix Ex Taq (2×conc.), 0.2 µl of ROX Reference

21 Dye (50X), 0.2µl of forward primer (10 µM), 0.2 µl of reverse primer (10 µM) and 0.2 µl of

22 TaqMan Probe (10 µM). Designs of PCR probes and primers were obtained from the Universal

23 Probe Library Assay Design Center at Roche (http://www.universalprobelibrary.com)

24 (Supplemental Table S1). Real-time amplifications were performed on the ABI PRISM 7900HT

25 Sequence Detection System (Applied Biosystems, Tokyo, Japan). The settings for the thermal

26 profile were an initial denaturation (30 s at 95°C) followed by 40 amplification cycles:

27 denaturation for 5 s at 95°C; annealing for 30 s at 60°C. Gene-specific standard curves were

28 generated using 2-fold serial dilutions of cDNA. The amount of target mRNA was expressed as

29 the ratio to *TBP* mRNA. Primers are listed in Table S1.

30

31 **Classification of genes**

The genes up-regulated or down-regulated significantly were categorized by panther process name of the gene prepared by Agilent data for human microarray.

Pathway analysis and statistical analysis

The genes up-regulated significantly were submitted to the KEGG pathway analysis at Kyoto University. Statistical analysis was performed with R (R Core Team; R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria; 2017 URL **https://www.R-project.org/**). For comparisons between groups, analysis of covariance (ANNOV method and t test for post hoc test) was used to assess the statistical significance of mean differences between groups, and pairwise t test was used for post hoc testing. The significance levels were set at $Pr(>F)$ 0.05 for anova, and $p < 0.05$ and $p < 0.01$ for post hoc testing.

DATA ACCESS

Microarray data of H_2O_2 treated cells is available in NCBI GEO data base with accession number GSE83369. (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE83369).

Microarray data of siRNA of NR4A3 treated cells is available in NCBI GEO data base with accession number GSE86924. (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE86924).

COMPETING INTEREST STATEMENT

CONFLICT OF INTEREST

No potential conflicts of interest relevant to this article were reported. Declarations of interest: none. The datasets generated during and/or analyzed during the current study are available from the corresponding author or author of E.U. upon reasonable request. The datasets are also available from NCBI GEO data base. Disclosure Statement: The authors have nothing to disclose.

1

8

9 **SUPPLEMENTARY DATA**

10 Supplementary Data are available.

11

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33

34 **TABLE AND FIGURES LEGENDS**

1

2   Table 1. Comparison of expression of genes measured by microarray and real time PCR

3   between H_2O_2 treated cells and control cells.

4   Average of 2 microarray data of H_2O_2 treated cells was compared with 1 microarray data of

5   control cells after normalization. For comparison with real time PCR, 12 RNA from 12 cell

6   culture dishes in 2 groups obtained for microarray were used, and the result of real time PCR

7   was expressed by the average of GAPDH and ß-actin. * $p < 0.05$

8   Table 2. Changes of gene expression after siRNA of NR4A3 treatment measured by microarray.

9   Average of 3 microarray data of siRNA of NR4A3 treated cells after normalization was

10   compared with average of 3 microarray data of control cells.

11

12   Fig.1. Effect of H_2O_2 concentration on the cell growth of 1.1B4 islet derived cell.

13   Islet derived 1.1B4 cell was seeded and cultured in RPMI-1640 medium, and 24 hours later cell

14   was added H_2O_2 to 10, 20, 30, 40, 50, 100, 200 µM and cell viability was analyzed at 24, 48 and

15   72 hours by MTT assay. (n=5)

16

17   Fig. 2. Effect of H_2O_2 concentration on *NR4A3* gene expression and time course of expression.

18   **A**, Human 1.1B4 cell (1.0×10^5 cells/ml) was incubated in 2ml culture medium and 24 hours

19   later medium was changed to individual concentration of H_2O_2 containing medium, then 4 or 24

20   hours later total RNA was extracted with Isogen. Expression of *NR4A3* was measured by real

21   time PCR with condition B. n=3. **B**, After addition of 100 µM of H_2O_2, cell was incubated

22   individual time and RNA was extracted. n=3. ß-Actin was used for control.

23

24   Fig. 3. Effect of *NR4A3* knocked down on the expression of *NR4A3* and cell viability. **A**, After

25   knocked down of *NR4A3* gene in 1.1B4 cell with siRNA, total RNA was extracted and

26   expression of *NR4A3* and ß-actin was measured by real time PCR with Cyber green method

27   with condition B. n=3 *$p < 0.05$. **B**, After knocked down of *NR4A3* gene of 1.1B4 cell, cell was

28   incubated with 100 µM of H_2O_2, and cell viability was measured by MTT assay. n=5. NC:

29   negative control; NFW: nuclease free water added cell; siNR4A3: siRNA of *NR4A3* treated cell.

17

1

2 Fig. 4. Changes of Various Gene expression in 1.1B4 Cell after the knocked down of *NR4A3*

3 mRNA measured by real time PCR.

4 (A) Human 1.1B4 cells were incubated 24hours, half of them (n=9) were added siRNA

5 (siNR4A3) and another half (n=9) were added nuclease free water (NFW), and after 52 hours,

6 mRNA was purified with RNeasy Plus Mini Kit. Expression of *NR4A3* was measured by real

7 time PCR with Taqman method using condition C. TATA binding protein (TBP) mRNA was

8 used for internal control. (B) The *NR4A1* expression, (C) *NR4A2* expression. (D) - (E)

9 *NFKB1*and *HMOX1* respectively. (F) *GLRX* expression (n=8). (G) Human 1.1B4 cells were

10 incubated 24hours, half of them (n=3) were added siRNA (siNR4A3) and another half (n=3)

11 were added NFW, and after 52 hours, mRNA was purified with Isogen reagent and treated with

12 RNase-free DNase. Expression of *SOD3* was measured by real time PCR with Cyber green

13 method using condition B. ß-Actin was used for control. (H) Expression of *GPX1* using same

14 method of (G). (I) *E2F1* using same method of (A) (n=8). (J) - (M) *p27Kip1*, *CDK2*, *CCNE1*

15 and *PMEL*, respectively, using same method of (A). (N) *RAB5B* using same method of (A)

16 (n=8).

17

18 Fig. 5. Gene structure of *HMOX1*.

19 Human *HMOX1* gene was obtained from PubMed gene (ACCESSION AY460337). NR4A3

20 binding motif of aaggtca were searched and abstract of the gene structure was displayed. NFkB

21 shows NFkB binding motif. FOSJUN shows FOS and JUN binding motif. EX1 *etc*. shows

22 exson 1 *etc*.

23

24