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Figure 1. Effect of PGE_2 on HIF-1α expression and nuclear localization in primary human breast ASCs. PGE_2 caused a significant increase in HIF-1α transcript (A) and nuclear protein expression (B). Confocal microscopy demonstrated that HIF-1α (*green*) is mainly perinuclear in breast ASCs under basal conditions (C, *top left*) and that PGE_2 stimulates the translocation of HIF-1α to the nucleus (C, *top right*). Treatment with DMOG (C, *bottom left*) and DMOG with PGE2 (C, *bottom right*) caused a much higher HIF-1α staining in the nucleus. The merged lamin B1+B2 nuclear stain (*red*) and HIF-1α are found as insets at the bottom right of each image. The percentage of cells positive for nuclear HIF-1α was also shown to be significantly increased with PGE_2 treatment (D). vc = vehicle control, n=3, repeated twice. Confocal images are representative of the majority of cells examined.

Figure 2. Role of HIF-1α in aromatase regulation. (A) ChIP demonstrated that PGE_2 and DMOG significantly stimulate the endogenous binding of HIF-1α to aromatase PII. Treatment of ASCs with PGE_2 or DMOG caused a significant increase in aromatase transcript expression (B) and activity (C). (D) HIF-1α overexpression in MCF-7 cells significantly increased aromatase activity in the presence of PGE_2. vc = vehicle control, n=3, repeated twice.

Figure 3. HIF-1α is necessary and acts cooperatively with CREB1 to induce PII activity in response to PGE_2. (A) A putative HRE (*italic*) was found to overlap with the proximal CRE (*boxed*) of aromatase PII. (B) Reporter assays demonstrated that mutation of the proximal CRE of aromatase PII inhibited the HIF-1α/DMOG-mediated effect on PII activity. (C) PII activity was significantly increased in HIF-1α transfected cells, treated with DMOG and PGE_2, and co-transfection with CREB1 resulted in a further increase in PII activity compared to cells transfected with either HIF-1α or CREB1 alone. Knockdown of HIF-1α (E) using siRNA significantly reduced PII activity (D) and aromatase transcript expression (F) and suppressed the PGE_2-mediated effect on PII activity (D). RLU: relative luciferase units, β-gal: β-galactosidase activity. n=3, all experiments repeated twice.

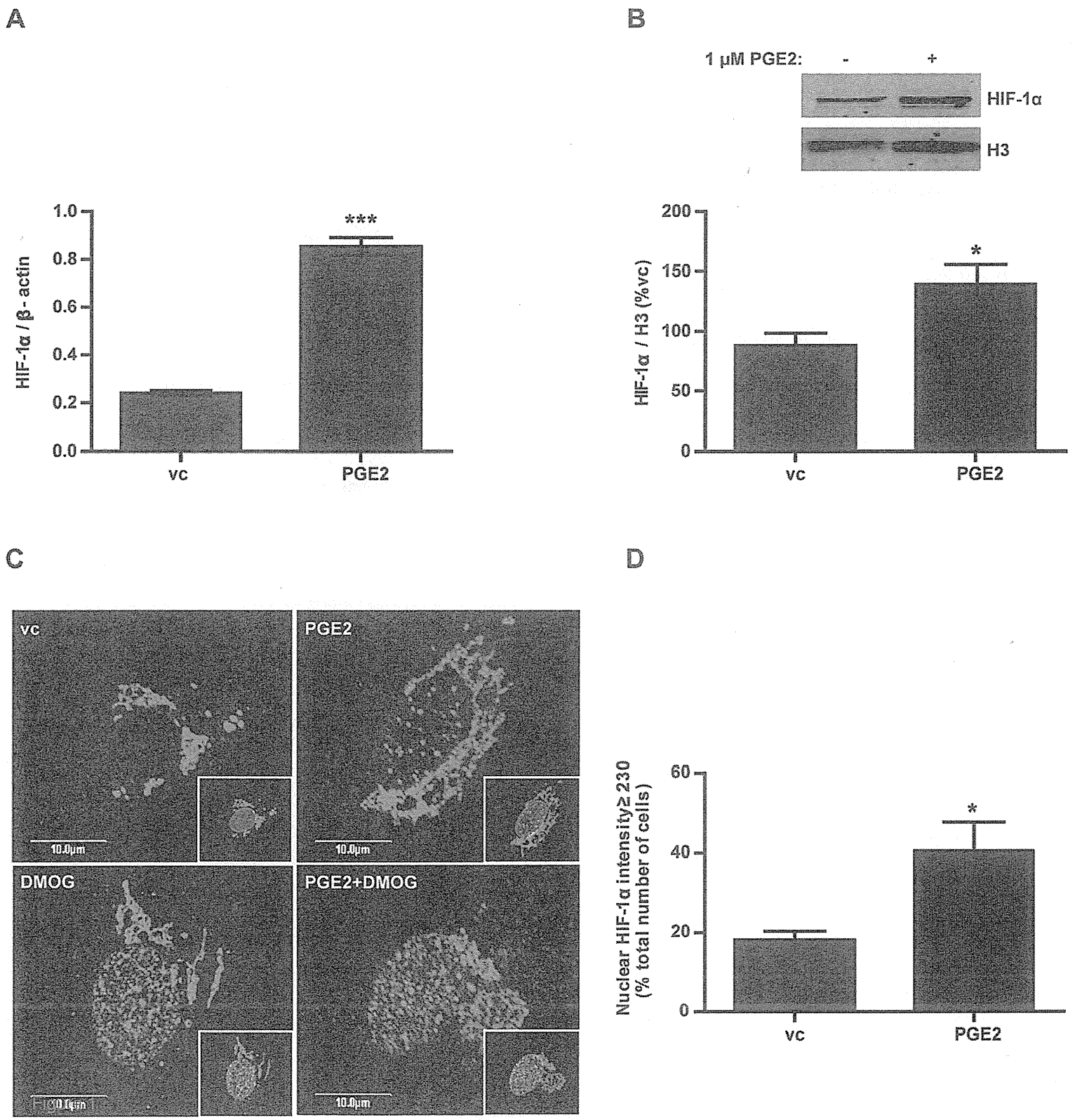
Figure 4. HIF-1α and aromatase expression in ASCs from tumor-bearing breast tissue compared to cancer-free using immunohistochemistry. (A) Percentage of HIF-1α positive ASCs from tumor-bearing breast tissue was significantly increased compared to tissue from cancer-free women. (B) Double-positive and single-positive ASCs for HIF-1α and aromatase were shown to be significantly increased in tumor-bearing compared to cancer-free tissues. Double negative ASCs for HIF-1α and aromatase were significantly reduced in tumor-bearing compared to cancer-free tissues. n = 10 for tumor-bearing breast tissue; n = 10/cancer-free breast tissue.

Figure 5. Model of the PGE_2-mediated regulation of aromatase expression by HIF-1α in breast ASCs. Tumor-derived factor PGE_2 increases HIF-1α transcript expression and nuclear localization. HIF-1α dimerizes with HIF-1β and then translocates to nucleus where it interacts with the proximal CRE of aromatase PII. HIF-1α together with CREB, CRTCs, CBP and p300 act to increase the PII-driven expression of aromatase.

Additional files

Additional file 1. Table S1: Clinicopathological data of breast cancer patients. Formalin-fixed and paraffin-embedded breast tissues from 10 Japanese breast cancer patients (IDC and DCIS), with differing hormone receptor status and grade used in Figure 4 are listed.

Additional file 2. Figure S1: Classification of ASCs for double immunohistochemistry. (A, B; *arrows*) HIF-1α and aromatase double positive cells. (C, D; *arrows*) HIF-1α positive and aromatase negative cells. (E, F; *filled arrows*) HIF-1α negative and aromatase positive cells. (E, *empty arrow*) HIF-1α positive and aromatase positive cell. (F, *empty arrow*) HIF-1α and aromatase double negative cell. Blue color in the cytoplasm as a result of Vector blue colorimetric reaction represents aromatase immunoreactivity while brown color in the nuclei as a result of DAB colorimetric reaction represents HIF-1α immunoreactivity.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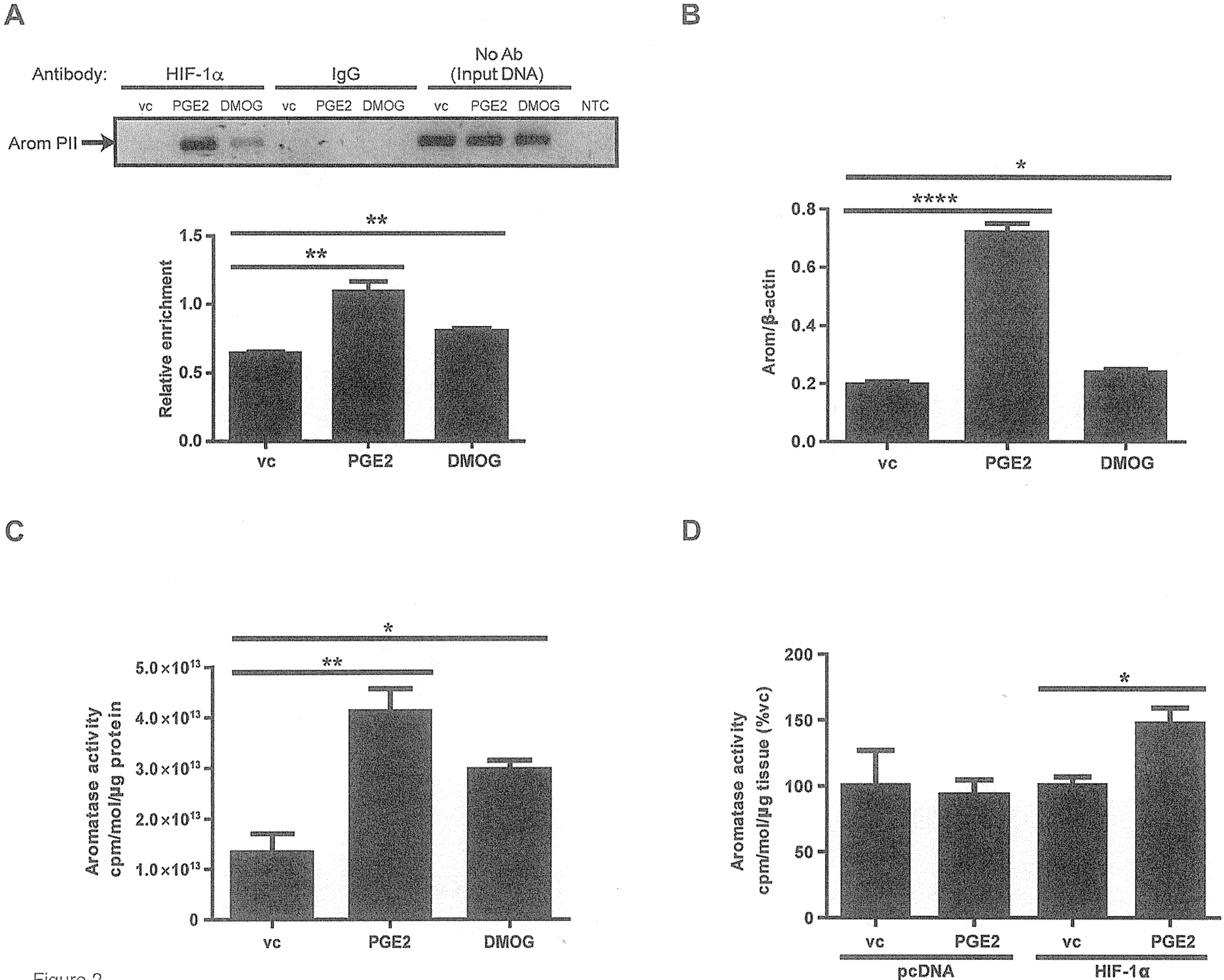
HIF-1α / β - actin
1.0
0.8
0.6
0.4
0.2
0.0
***
vc
PGE2
B
1 μM PGE2:
-
+
HIF-1α
H3
HIF-1α / H3 (%vc)
200
150
100
50
0
*
vc
PGE2
C
vc
PGE2
DMOG
PGE2+DMOG
10.0μm
D
Nuclear HIF-1α intensity≥ 230
(% total number of cells)
60
40
20
0
*
vc
PGE2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Antibody:
HIF-1α
IgG
No Ab (Input DNA)
vc
PGE2
DMOG
vc
PGE2
DMOG
vc
PGE2
DMOG
NTC
Arom PII
Relative enrichment
1.5
1.0
0.5
0.0
**
**
vc
PGE2
DMOG
B
*
****
Arom/β-actin
0.8
0.6
0.4
0.2
0.0
vc
PGE2
DMOG
C
*
**
Aromatase activity cpm/mol/μg protein
5.0×10^13
4.0×10^13
3.0×10^13
2.0×10^13
1.0×10^13
0
vc
PGE2
DMOG
D
Aromatase activity cpm/mol/μg tissue (%vc)
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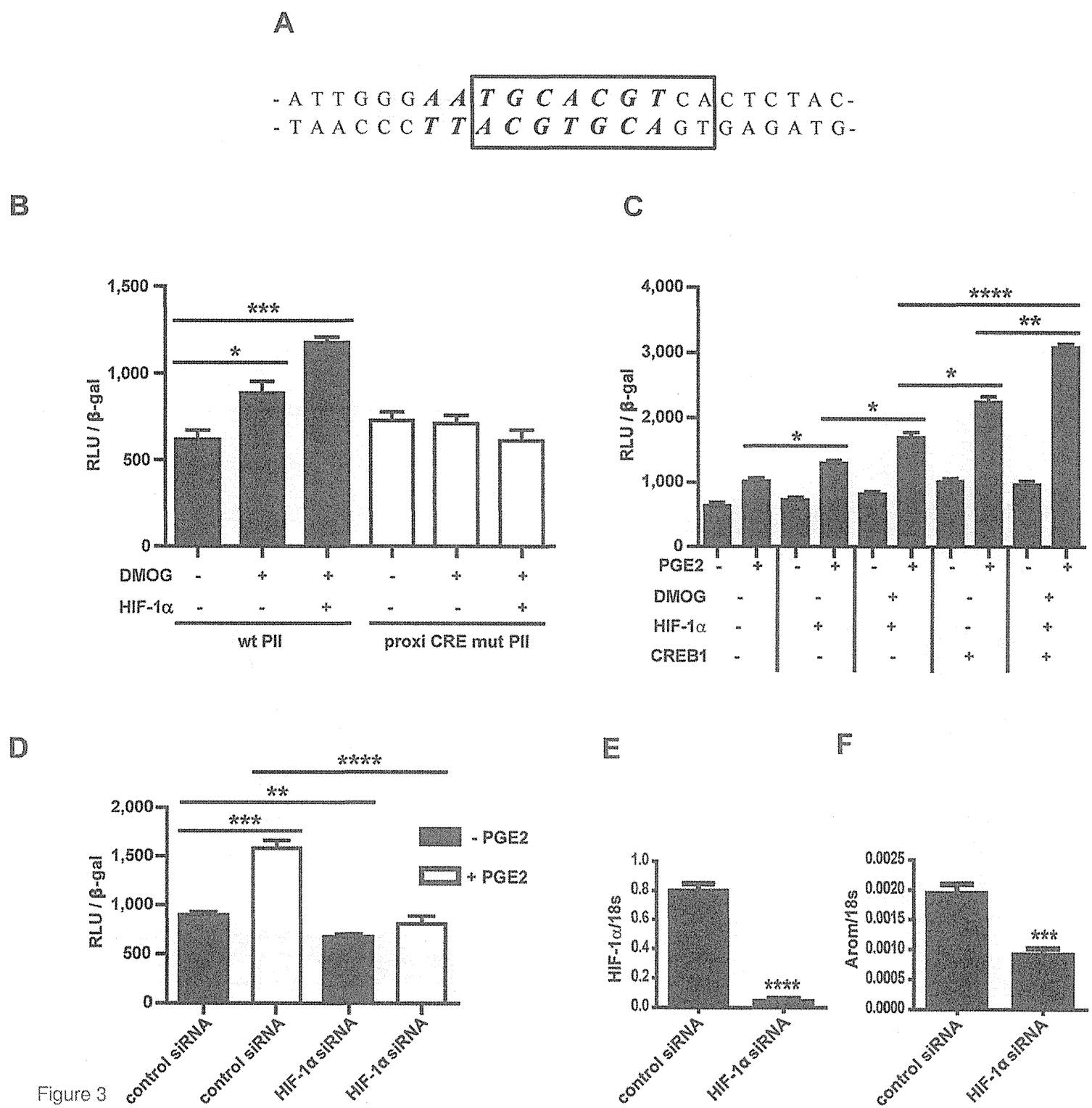
150
100
50
0
*
vc
PGE2
vc
PGE2
pcDNA
HIF-1α

Figure 2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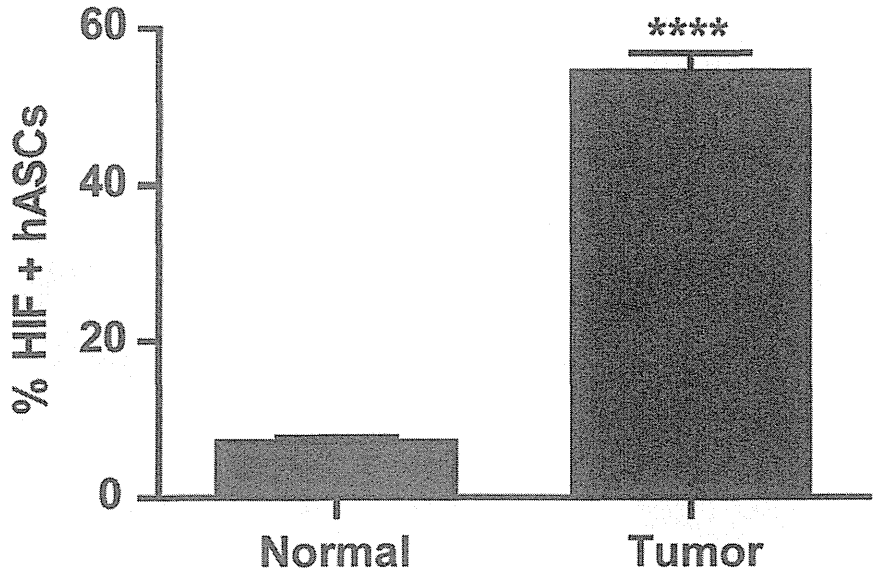
B
RLU / β-gal
1,500
1,000
500
0
***
*
DMOG
HIF-1α
wt PII
proxi CRE mut PII
C
RLU / β-gal
4,000
3,000
2,000
1,000
0
****
**
*
PGE2
DMOG
HIF-1α
CREB1
D
RLU / β-gal
2,000
1,500
1,000
500
0
****
**
***
- PGE2
+ PGE2
control siRNA
control siRNA
HIF-1α siRNA
HIF-1α siRNA
E
HIF-1α/18s
1.0
0.8
0.6
0.4
0.2
0.0
****
control siRNA
HIF-1α siRNA
F
Arom/18s
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
***
control siRNA
HIF-1α siRNA

Figure 3

A


60
40
20
0
% HIF + hASCs
****
Normal
Tumor

B

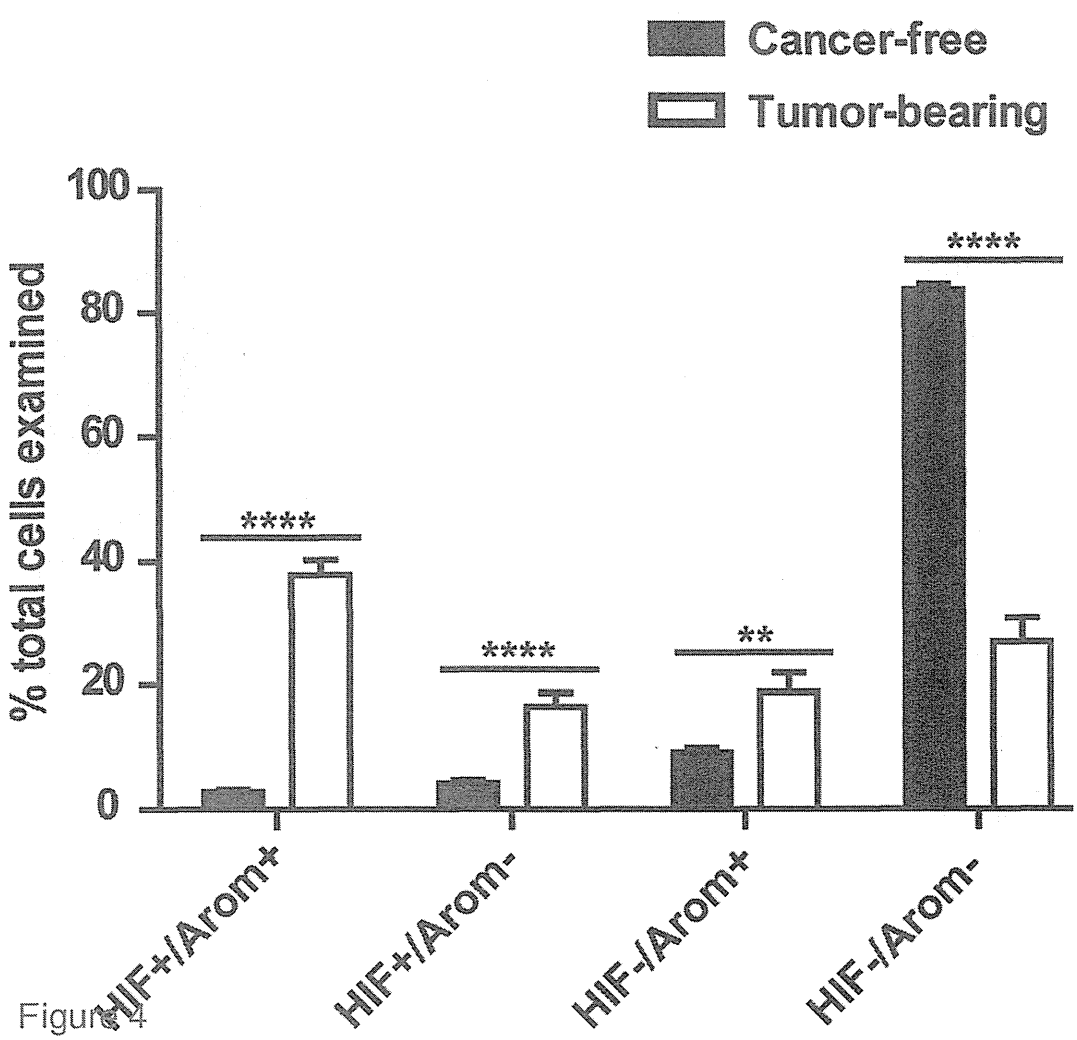
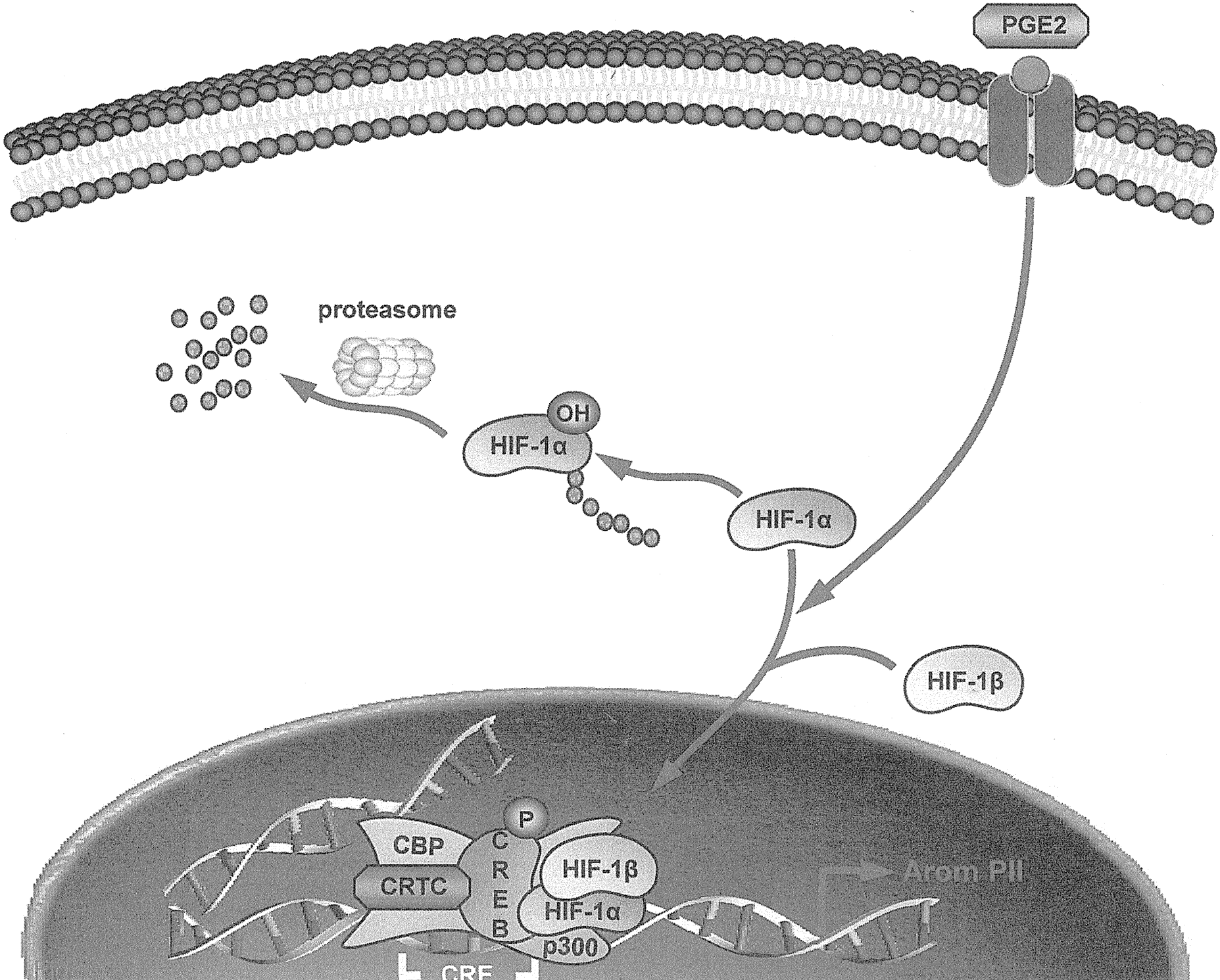

Cancer-free
Tumor-bearing
100
80
60
40
20
0
% total cells examined
****
****
**
****
HIF+/Arom+
HIF+/Arom-
HIF-/Arom+
HIF-/Arom-

Figure 4


PGE2
proteasome
OH
HIF-1α
HIF-1α
HIF-1β
P
CBP
CRTC
C
R
E
B
HIF-1β
HIF-1α
p300
CRE
Arom PII

Additional files provided with this submission:

Additional file 1: Additional file 1, Table S1_Breast Cancer Res.doc, 36K
http://breast-cancer-research.com/imedia/1323609701963082/supp1.doc
Additional file 2: Additional file 2, Figure S1_Breast Cancer Res.tif, 18495K
http://breast-cancer-research.com/imedia/1719921982963081/supp2.tiff

Cancer Science
The official journal of the Japanese Cancer Association JCA

Androgenic pathway in triple negative invasive ductal tumors: Its correlation with tumor cell proliferation

Keely M. McNamara,[1,6] Tomomi Yoda,[1] Yasuhiro Miki,[1] Niramol Chanplakorn,[1,2] Sansanee Wongwaisayawan,[2] Pimpin Incharoen,[2] Youwanush Kongdan,[3] Lin Wang,[4] Kiyoshi Takagi,[1] Takagi Mayu,[1] Yasuhiro Nakamura,[1] Takashi Suzuki,[1] Noriko Nemoto,[1,5] Minoru Miyashita,[1,5] Kentaro Tamaki,[1,5] Takanori Ishida,[5] Noriaki Ohuchi[5] and Hironobu Sasano[1]

[1]Department of Pathology, Tohoku University School of Medicine, Sendai, Japan; Departments of [2]Pathology; and [3]Surgery, Ramathibodi Hospital, Bangkok, Thailand; [4]Department of Physiology, Harbin Medical University Daqing Branch, Daqing, China; [5]Department of Surgery, Tohoku University School of Medicine, Sendai, Japan

(Received November 20, 2012/Revised January 21, 2013/Accepted January 23, 2013/Accepted manuscript online February 1, 2013/Article first published online March 15, 2013)

Triple negative breast cancer (TNBC) is defined by estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 negativity. Patients with TNBC frequently undergo an aggressive clinical course due to the unavailability of specific targeted therapies. Androgen receptor (AR) was reported to be expressed in up to 60% of TNBC cases but there have been controversies as to the roles of androgen signaling through AR in TNBC. Therefore, in this study, we analyzed the status of AR in combination with androgen synthesizing enzymes (5α-reductase type 1 (5αR1) and 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5)] in order to further understand androgenic actions in TNBC. Androgen receptor, 5αR1, and 17βHSD5 were immunolocalized in a cohort of 203 TNBC patients from Thailand and Japan. We then correlated the findings with clinicopathological characteristics (age, stage, tumor diameter, lymph node invasion, metastatic spread, Ki-67 labeling index, disease-free survival, and overall survival) of the patients. Univariate analysis revealed that AR+/enzyme+ cases were associated with a significantly lower Ki-67 labeling index than AR−/enzyme− samples. Multivariate analysis indicated the presence of significant positive correlations between AR and enzyme status in tumor cells, and between tumor diameter, lymph node invasion, and distant metastasis. Significant negative correlations were also detected between Ki-67 labeling index and AR status ($P = 0.04$) or 5αR1 ($P < 0.001$). Cox proportional hazards analysis showed that Ki-67 labeling index and stage were the only factors predicting disease-free and overall survival of the patients, although univariate Kaplan–Meier analysis revealed AR/5αR1 negativity suggested a more adverse clinical course up to 80 months after surgery. These results suggest that the presence of androgen synthesizing pathways in addition to AR expression in tumor cells could confer a better clinical outcome through suppression of cell proliferation. (*Cancer Sci* 2013; 104: 639–646)

Breast cancer is the most common malignancy in women[1] and, although recent advances in clinical management have significantly improved the survival rates of the great majority of breast cancer patients,[2] one subtype, so-called triple negative breast cancer (TNBC) continues to be associated with an adverse prognosis.[3] Triple negative breast cancer is characterized by the absence of estrogen receptor-α (ERα), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression in the tumor cells and constitutes approximately 6–60% of all breast cancer cases, depending on the cohort evaluated.[4–23] This subtype is considered to be far more diverse compared to other subtypes of breast malignancy.[24–26] Triple negative breast cancer is generally associated with relatively adverse clinical outcome[27–30] primarily due to the lack of specific therapies, higher rates of tumor cell proliferation, and more aggressive behavior.[31]

Numerous published studies have attempted to identify biomarkers that could further subclassify TNBC into disease subtypes. For instance, growth factors such as epidermal growth factor receptor[32] and insulin-like growth factor-1[33] have been studied and higher expression was reported to be associated with adverse clinical outcomes in TNBC patients. Other biomarkers reported so far include Numb protein,[34] chromosomal instability,[35] EZHR,[36] and miR34b.[37] Among these markers, one of the most extensively investigated but also one of the most controversial is the androgen receptor (AR).

The AR in triple negative breast tumor cells has been reported to be expressed at a relatively lower rate than in other types of breast cancer or even in breast cancer as a whole (50–100% in non-subtype specific[38–43]; 0–53% in TNBC[4–19,21,22,44,45]). However, the mere presence of AR in even a subset of TNBC patients suggests the manipulation of androgenic pathways in tumor cells could serve as a therapeutic option, at least for some TNBC patients. In addition, the availability of AR targeted agents, developed primarily for the treatment of prostate cancer, potentially makes the manipulation of androgenic pathways in TNBC patients more appealing as such treatment could be incorporated into clinical practice with less difficulty compared to other modes of target-specific therapies. The use of such therapies, however, is dependent on a clear understanding of the role of androgenic pathways in the biological behavior of TNBC.

The biological roles of androgens in TNBC are in dispute. Immunohistochemical studies looking at the presence of AR in tumor cells have reported conflicting results in terms of the correlation between AR and clinical outcome; some studies indicated a survival advantage,[11,46,47] others showed no significant effects of AR expression in tumor cells on survival of the patients.[20,43,48] However it is also true that, in contrast to these clinical studies, results of *in vitro* investigations consistently showed that AR may replace ER and PR as a driver of tumor proliferation and growth in TNBC cell lines.[48–57] The suggestion that AR expression could be an adverse marker is partially supported by one clinical study using gene expression profiles rather than immunoreactivity to define AR+ TNBC using

[6]To whom correspondence should be addressed.
E-mail: kmcnamara@patholo2.med.tohoku.ac.jp

doi: 10.1111/cas.12121

luminal androgen receptor (LAR) gene expression profiles.[56] Based on these findings, clinical trials of AR antagonist have been initiated,[58] but further investigations are considered necessary to establish the precise roles of AR in TNBC patients.

We previously reported that AR expression in tandem with the presence of androgen synthesizing enzymes could eventually determine the clinical outcome for breast cancer patients as a whole, regardless of their intrinsic subtypes.[59] Therefore, we examined AR and androgen metabolizing enzymes in whole tissue sections of a cohort of 203 triple negative surgical breast cancer specimens obtained from both Japanese and Thai cohorts in order to evaluate the influence or effects of AR status in tumor cells on the Ki-67 labeling index of tumor cells, as well as on overall survival (OS) and disease-free survival (DFS) of the patients, in order to further extrapolate our findings on the receptor and enzymes in this study.

Materials and Methods

Patient cohorts. Following approval from the relevant institutional review boards or ethical committees (Japan, Tohoku University School of Medicine ID: 2012-1-185; Thailand, Mahidol University, Faculty of Medicine Ramathibodi Hospital ID: 01-54-50), archival materials of TNBC patients were retrieved from the surgical pathology files. The status of triple negativity in tumor cells was confirmed by reviewing the ER/PR/HER2 stained slides based on American Society of Clinical Oncology/College of American Pathologists guidelines. From these cohorts, a total of 203 TNBC specimens with whole tissue availability (Japan, 86 cases; Thailand, 117 cases) were examined. Clinical data including patient age, stage, tumor diameter, lymph node involvement, and metastatic spread was available in both cohorts after review of the charts of individual patients.

Immunohistochemistry. Archival tissue blocks were serially sectioned at a thickness of 4 μM and placed on pre-cleaned glue-coated glass slides for immunohistochemistry (IHC). Immunostaining of serial tissue sections for AR, 5α-reductase type 1 (5αR1) and 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5) was carried out as described previously.[59,60] In brief, antibodies against all three targets (AR, AR441 1:50 [Dako, Carpinteria, CA, USA]; 5αR1, 1:1000, provided by Dr D.W. Russell [University of Texas Southwestern Medical Center, Dallas, TX, USA]; 17βHSD5, NP6.G6.A6 1:200 [Sigma, St. Louis, MO, USA]) were used in conjunction with a streptavidin–biotin visualization method (Histofine kit; Nichirei, Tokyo, Japan). A control tissue (AR, prostate; 5αR1, liver; 17βHSD5, adrenal) was included in all runs of immunostaining in order to confirm the specificity of immunostaining. Ki-67 immunostaining was carried out as described previously.[59]

Evaluation of immunoreactivity. Immunoreactivity was evaluated as follows. In an evaluation of AR in tumor cells, immunoreactivity was assessed by the H score.[61] In brief, the H score was obtained by assessing immunointensity (on a scale of 0–3) and prevalence in 100 cells over five different areas throughout the tumor (scale, 0–300). Cytoplasmic (5αR1, 17βHSD5) immunoreactivity was evaluated using a semiquantitative scale (0–2) which divided tumor cells into categories: no immunoreactivity; 0.1–50% immunoreactivity; and 50.1–100% immunoreactivity.[60] All slides were counted three times in order to assess intra-observer variability and variation was found to be less than 10%. In addition, a subset of tissue slides was assessed by at least two of the authors (KM, YN, KT) in order to assess inter-observer variability and inter-observer differences. Variation for the H score was less than 12%, and for the enzyme score less than 5%. For Ki-67 more than 500 tumor cells were counted in each case at the sites of hot spots. Hot spots were identified after evaluating the stained slides at low magnification. The immunoreactivity was quantified using a labeling index (LI). All the results were assessed by two authors (TY, HS) in order to assess reproducibility of the data, and found to be in agreement. When dichotomous variables were needed we used a cut-off point of 10% LI (converted from the H score) for AR, a score of 2 (>50% positivity) in enzyme staining.

Statistical analysis. Statistical analyses were carried out using JMP software (JMP Pro 9.0.2; SAS Institute, Tokyo, Japan). To assess the overlap between enzymes and receptor expression, as well as significant differences between categorical variables by country, a χ^2-test was used. Differences in linear variables between two different cohorts were tested using Student's *t*-test. Analysis of the effect of AR and enzyme expression on the Ki-67 LI was tested by stratifying the groups by AR/5αR1 status and using ANOVA followed by the Tukey–Kramer HSD *post-hoc* test. Correlation analysis was carried out using the Pearson correlation in order to compare the correlations among AR, enzyme expression, and clinical and pathological markers. Interactions between receptor and enzyme status and survival were tested using a multivariate Cox proportional hazards model, and statistical significance of individual factors examined was tested using Kaplan–Meier survival analysis.

Results

Androgen receptor, 5αR1, and 17βHSD5 in TNBC tumor cells. Androgen receptor immunoreactivity was detected in the nuclei of tumor cells and 17βHSD5 and 5αR1 immunoreactivity was detected in the cytoplasm of tumor cells. Immunostaining in serial tissue sections indicated that in the great majority of the cases examined, AR, 5αR1, and 17βHSD5 immunoreactivity, where present, was detected in comparable areas (Fig. 1).

Differences in clinicopathological parameters between cohorts. In order to asses any possible differences between the two separate cohorts, χ^2 assessment of the distribution of values between the Thai and the Japanese cohorts was undertaken. The number of patients and the percentage of populations (brackets) are given in Table 1. Immunoreactivity of 17βHSD5, lymph metastasis, and distant metastasis did not vary between Japanese and Thai cases but other factors such as age, stage, Ki-67, and tumor diameter were significantly different.

Prevalence of AR, 5αR1, and 17βHSD5 in TNBC patients. In 203 TNBC cases examined in this study, positive AR immunoreactivity in the tumor cells (defined as >10% LI) was detected in 51 cases (25%). The immunoreactivity of 5αR1 and 17βHSD5 in breast cancer cases was greater than that of AR, with 71.7% of cancers positive for 5αR1 and 69.8% for 17βHSD5. There were significant differences between the Thai and Japanese cohorts in terms of AR positivity, AR H score, and 5αR1 score ($P < 0.03$) but not in 17βHSD5 score ($P = 0.61$). These results are summarized in Table 1.

Correlation between AR, enzymes, and clinicopathological factors in TNBC. Using the χ^2-test, the status of AR and 17βHSD5 in tumor cells was significantly correlated ($P = 0.001$) (either double negative or double positive), and also that of AR and 5αR1 ($P = 0.04$) in the whole cohort. A significant positive correlation was also detected between these two enzymes in the whole cohort ($P = 0.001$, 53% of samples showing the same enzymatic score, 70% when classified as negative or positive). In the whole cohort of patients, 40% were receptor and enzymes double negative (AR−/5αR2−/17βHSD5−) and 8% were receptor/enzymes double positive (AR+/5αR2+/17βHSD5+) using a cut-off value of 2 (>50% immunoreactivity) for the enzymes and 10% LI for AR.

Pearson's correlation was used to assess the strength (Pearson's Rho; Table 2, first line for each parameter), and the significance (*P*-value; Table 2, second line for each parameter, values in parentheses) of correlations between various histologi-

doi: 10.1111/cas.12121

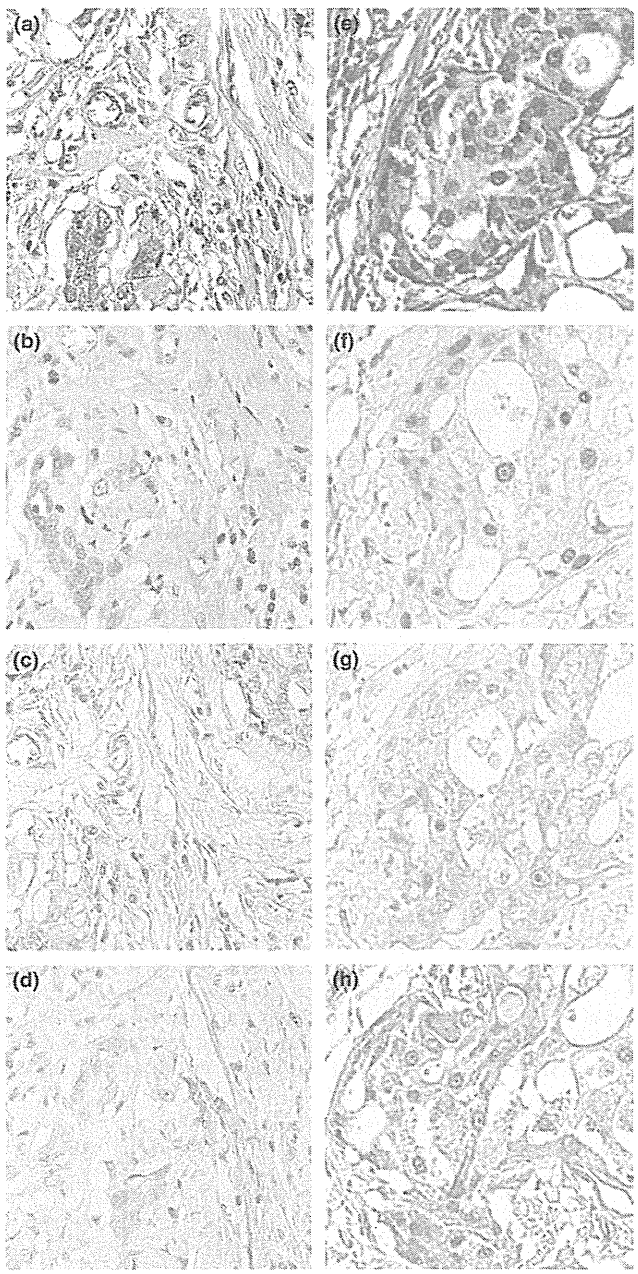


Fig. 1. Representative photographs of androgen receptor (AR), 5α-reductase type 1 (5αR1), and 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5) immunohistochemistry in AR−5αR1−17βHSD5− (a–d) and AR+5αR1+17βHSD5+ (e–h) triple negative breast carcinomas. Hematoxylin–eosin staining (a,e). Androgen receptor was immunolocalized in the nuclei of carcinoma cells at variable immunoreactivity (b,f). Both 5αR1 (c,g) and 17βHSD5 (d,h) were immunolocalized in the cytoplasm of carcinoma cells. Stromal cells were negative in areas either adjacent or distal to carcinoma. Original magnification, x200.

cal and clinical parameters. Table 2 shows strong and significant (indicated in bold italics) or near significant (bold) correlations found between androgenic pathways as well as between androgenic pathways and indicators of tumor proliferation (AR and 5αR2, $P = 0.002$; AR and 17βHSD5, $P = 0.001$; AR and age, $P < 0.001$; 5αR1 and 17βHSD5, $P < 0.0001$), and also between the three clinicopathological factors used to define

Table 1. Summary of distribution of clinicopathological features in Thai, Japanese, and combined cohorts of patients with triple negative breast cancer ($n = 203$)

	Thai, *n* (%)	Japanese, *n* (%)	Combined	*P*-value
5αR1				
Negative	26 (22.2)	31 (36.4)	57 (28.2)	**0.03**
<50% positivity	28 (23.9)	23 (27.0)	51 (25.2)	
≥50% positivity	63 (53.8)	31 (36.4)	94 (46.5)	
17βHSD5				
Negative	34 (29.1)	27 (31.1)	61 (30.3)	0.61
<50% positivity	53 (45.2)	42 (48.8)	95 (46.7)	
≥50% positivity	30 (25.6)	17 (19.7)	47 (23.1)	
AR				
Positive (>10% LI)	20 (17.1)	31 (36.1)	51 (25.1)	0.002
Negative (≥10% LI)	97 (82.9)	55 (63.9)	152 (74.9)	
TNM stage				
I	32 (27.8)	30 (37.0)	62 (31.6)	**0.01**
IIA	46 (40.0)	27 (33.3)	73 (37.2)	
IIB	11 (9.6)	8 (9.9)	19 (9.7)	
IIIA	14 (12.2)	6 (7.4)	20 (10.2)	
IIIB	9 (7.8)	2 (2.4)	11 (5.6)	
IIIC	1 (0.9)	8 (9.9)	9 (4.6)	
IV	2 (1.7)	0 (0)	2 (1.0)	
Tumor size				
<20 mm	38 (33.9)	46 (56.7)	84 (43.5)	**0.005**
20.1–50 mm	63 (56.2)	31 (38.3)	94 (48.7)	
>50.1 mm	11 (9.8)	4 (4.9)	15 (7.7)	
Lymph invasion				
No	74 (65.5)	50 (62.5)	124 (64.3)	0.62
Yes	39 (34.5)	30 (37.5)	69 (35.8)	
Presence of distant metastasis				
Yes	2 (1.7)	0 (0)	2 (1.0)	0.23
No	113 (98.3)	81 (100)	194 (99.0)	
Age				
<50 years	59 (50.4)	29 (33.3)	88 (43.1)	0.04
≥50 years	58 (49.6)	58 (66.7)	116 (56.9)	
Ki-67				
<25%	75 (63.6)	30 (37.0)	105 (52.8)	**0.003**
≥25%	43 (36.4)	51 (63.0)	94 (47.2)	

Bold indicates significant value. 5αR1, 5α-reductase type 1; 17βHSD5, 17β-hydroxysteroid dehydrogenase type 5; AR, androgen receptor; LI, labeling index.

TNM breast cancer stage (tumor diameter, lymph node invasion, and metastatic spread). Significant negative correlations were also detected between Ki-67 LI and AR ($P = 0.048$) or 5αR1 ($P = 0.004$) status in tumor cells. The AR status tended to be inversely correlated with tumor diameter but this correlation did not reach statistical significance ($P = 0.055$). When stratified by country, the correlation coefficients obtained showed similar trends but not all significant associations remained.

In order to further assess the effects of AR/enzyme action on Ki-67 LI in tumor cells in TNBC cases, we subclassified the cases according to the AR/5αR1 status and compared the Ki-67 LI among these different groups of TNBC patients. We did not include 17βHSD5 in this stratification because of the relatively small cohort, and the close correlation between 17βHSD5 and 5αR1. In this analysis AR+/5αR1+ cases had the lowest Ki-67 LI and AR−/5αR1− the highest Ki-67 LI. (Fig. 2). The tendency among the four different groups still remained even if the Thai and Japanese cohorts were analyzed