度や形態像との比較を行い、末梢血における間葉系幹細胞の生物学的意義についても検討する必要があると考えられた。

結論

成人ヒト抹消血中から間葉系幹細胞を採取することが可能であることを明らかにした。採取された間葉系幹細胞の性状について今後の検討が必要と考えられた。

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厚生科学研究費補助金　（ヒトゲノム・再生医療等研究事業）
分担研究報告書

「月経血・臍帯血・末梢血由来間葉系細胞の分化機構に関する分子レベルでの解明」

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研究要旨　骨髄間葉系細胞のstemnessの維持機構に必要な分子メカニズムの解明を目的とした。カドヘリン分子のclass スイッチの現象がmesenchymal epithelial transformation を誘導する機構と考えており、cadherin分子の分子間結合を調節するメカニズムについて検討した。低分子量GTP結合蛋白質Rap1がcdherin分子間の結合を増強してstemnessの維持を制御している可能性が考えられた。

A. 研究目的

骨髄・臍帯血・月経血間葉系細胞の多分化能の維持機構の明らかにすることで、多分化能を維持したまま細胞を増殖させるシステムを構築する必要がある。こたため、本研究分担では、多能性を維持し続ける機構を明らかにする。とくに、mesenchymal-epethelial transformationはこれまで癌化に重要なメカニズムと考えられており、stemnessの維持に重要な現象であることが予想される。本分担研究者は特にcadherin分子が骨髄内でadult hemangioblastの維持に重要で、ここでVascular endothelial cadherin (VE-cadherin)の発現がおきることで血管内皮細胞への分化が誘導される現象を明らかにしていた。つまり、cadherin分子のスイッチ(N-cadherin→VE-cadherin)が重要であると考えた。このため、cadherinの分子間結合の減弱がstemnessに影響を及ぼすと考え、cadherinの分子間調節機構を明らかにする。

B. 研究方法

① VE-cadherinプロモーター依存性にGFPを発現するマウスの作製　(VE-cad/EGFP)—

血管内皮細胞に分化する骨髄内細胞としてこれまでにEndothelila progenitor cell (EPC)が提唱されている。この細胞を骨髄内でマーキングして骨髄内での動きや、末梢血液へのmobilizationをモニターすることでstemnessの維持機構を予想できると考えた。VE-cadherin プロモーターの下流にCre recombinaseを発現するマウスを作製して、このマウスとEGFP レポーターマウスを交配してVE-cadherin依存性にEGFPを発現するマウスを作製した。

② Cadherin依存性の接着制御メカニズムの検討—

cAMPはこれまでPKA依存性にRap1を活性化すると考えられていた。血管内皮細胞をFolskolin, 、dibutylic cAMPで刺激したときの細胞接着を検討する。また、cAMP-Epac-Rap1系がCadherin依存性の細胞接着に重要かどうかを調べた。

③ VE-cadherin 依存性GFP発現マウスの骨髄の組織学的検討ならびにFACS解析—

VE-cadherinによるGFPの発現は骨髄を固定して抗GFP抗体で染色して検討した。また、骨髄のGFP発現細胞を大腿骨から収集し、GFP発現細胞をソーティングした後、FACSで表面分子マーカーを調べた（VE-cadherin, CD34, Lin, CD45, CD45, CD31）。

④ VE-cadherin依存性の細胞接着—血管内皮細胞のVE-cadherin依存性の細胞間接着のメカニズムを明らかにするために培養ヒト血管内皮細胞を使用した。また、VE-cadherinの細胞外ドメインだけを培養皿にコーティングしてVE-cadherinへの細胞接着を検討することでVe-cadherinによる接着能を調べた。

C. 研究結果

VE-cadherinプロモーター依存性にGFPを発現するマウスはEPCあるいはvascular progenital cell (VPC)を骨髄で同定できる：

VE-cad/EGFPマウスを観察したところ生後５週

で血管のGFP陽性細胞が消失していた。このマウスが妊娠あるいは心筋梗塞などの虚血による血管新生シグナルが個体に生ずると骨髄内にGFP陽性細胞が出現することがわかった。

さらに虚血部位の血管内皮細胞と血管平滑筋細胞にGFP陽性細胞が取り込まれていることから、これらの細胞が骨髄からstemnessを解除されて分化して、血管構築細胞になったと考えられた。

骨髄の血管内皮細胞もVE-cadherinを発現する：VPCあるいはEPCへと分化した細胞が骨髄から離れるためには(骨髄のnicheから放出される)、nicheからの遊離促進メカニズムがあると考えられている。本研究では、VE-cadherinの陽性細胞が骨髄血管内皮細胞にも出現することから、血球として存在していたEPC, VPCが骨髄内血管内皮細胞とVE-cadherin依存性の細胞間接着を介して末梢血液への流入が可能となったと考えている。実際VE-cad/EGFPマウスに心筋梗塞を作製して骨髄を調べると既存の血管の内皮細胞がGFP陽性つまりVE-cadherinを発現するようになっていた。

血管内皮細胞同士の接着はcAMPシグナルを介してVE-cadherin依存性の接着を介して安定化している：

一度血管内皮細胞に分化してしまうと、多分化能を失うと考えられているが、発生の段階では心臓でendothelial mesenchymal transformationがおきて、弁を構築することもわかっている。しかし、成熟した段階では多分化能を失い、内皮細胞としての機能を維持している。細胞間接着は特に重要であり、細胞の周囲の環境特に細胞間接着によってもたらされる情報伝達系が重要である。cAMPを増加させる刺激では細胞間接着が増強されるが、このメカニズムは明らかでなかった。cAMPがEpacを活性化してRap1の活性化が細胞間接着でおきて、VE-cadherinのホモフィリックな結合が増強させることがあきらかになった。

D. 考察

多分化能を維持に不可欠な因子として生体内ではnicheに存在することが考えられている。特に骨髄内のhematopoietic stem cellやmesenchymal cellは細胞間接着があることでそのstemnessを維持していると考えられる。

Hematopoietci stem cellはosteoblstとの接着をN-cadherinを介しておこしていることでstemnessが維持されていると考えられている。われわれの心筋梗塞もでるではEPCあるいはVPCでN-cadherin依存性の細胞間接着が、VE-cadherinを発現することで接着が切れて、骨髄でのniche環境から、遊離してstemnessの維持が不可能になるという現象がおきていると考えられた。骨髄間葉系細胞の培養を継続するときに細胞間接着を維持したまま培養をするべきかどうかは大きな検討課題であると考えられた。特にフィーダー細胞との接着が重要なのか、フィーダー細胞からの分泌される因子が重要なのかが問題となる。今回われわれの結果から培養条件でniche環境を再現できればstemnessの維持ができることが示唆された。特に骨髄間葉系細胞の細胞間接着因子を明らかにして、cadherinのスイッチがおきないような条件で維持することが重要であると考えられた。

N-Cadherin依存性の接着はアンギオポエチンが維持しているという報告がなされた。アンギオポエチンはTie２受容体を介したシグナルを送っているが、われわれはアンギオポエチンがRap１を活性化するという結果も得ている。また、VE-cadherin同士の接着もRap1により増強されるという結果からRap1が
が細胞間接着を亢進させるシグナルを送っていると考えられた。

心筋梗塞や妊娠で分泌される因子がN-cadherinによる接着でのnicheを阻害して、分化の促進方向に向かわせることから、今後こういった未同定の因子を排除することで培養条件下でのstemness維持の培養が可能になると考えられた。

来年度にかけて、骨髄間葉系細胞に発現する未同定受容体やフィーダー細胞から分泌される未同定因子をsignal sequence trapを用いて検討していく計画である。

E. 健康危険情報

なし。

F. 結論

細胞間接着がstemnessの維持に重要であり、cadherinの接着が特にniche環境を形成するのに機能していることがわかった。また、このcadherinの接着はRap1が制御していることがわかった。

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2. 学会発表

特になし。

H. 知的財産権の出願・登録状況

特になし。

Ⅲ　研究成果に関する一欄表

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Ⅳ　研究成果の刊行物・別冊

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Cancer Letters xx (2004) 1–8
CANCER Letters
www.elsevier.com/locate/canlet

Effects of 3-methylcholanthrene on the transcriptional activity and mRNA accumulation of the oncogene *hWAPL*

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Received 28 April 2004; received in revised form 26 July 2004; accepted 5 August 2004

Abstract

hWAPL is a human oncogene associated with uterine cervical cancer. Here, we demonstrate that *hWAPL* transcription is induced by 3-methylcholanthrene (3-MC) in the cervical carcinoma-derived cell line SiHa. *hWAPL* transcription was analyzed with evaluation of the mRNA and heterogeneous nuclear RNA (hnRNA) levels by quantitative real time PCR analysis. Flow cytometric analysis suggested that the alteration of *hWAPL* mRNA levels is independent of cell cycle profile. We also found that DMSO and some components of FBS affect *hWAPL* transcription. Interestingly, when the aryl hydrocarbon receptor (AhR) function was inhibited by α-naphthoflavone (ANF), the induction of *hWAPL* transcription by 3-MC was greater than that in AhR-functioning normal cells. These observations suggest that there are complex mechanisms regulating the transcription of *hWAPL*. Furthermore, mRNA level of a mouse homolog of *hWAPL* in mouse uterus was induced by 3-MC injection into the abdominal cavity. Thus, some effects from 3-MC exposure on uterus may be mediated by the unscheduled overexpression of *hWAPL*.

Keywords: 3-Methylcholanthrene (3-MC); hWAPL; Uterine cervical cancer; Aryl hydrocarbon receptor (AhR); α-naphthoflavone (ANF)

1. Introduction

Previously, we have isolated and characterized a novel human gene termed *hWAPL* [1]. Our initial observations suggested that *hWAPL* expression is associated with uterine cervical cancer, although the mechanism was not clear. *hWAPL* is the human homolog of the *wings apart-like* (*wapl*) gene in

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0304-3835/$ - see front matter © 2004 Published by Elsevier Ireland Ltd.
doi:10.1016/j.canlet.2004.08.006

Drosophila melanogaster. The protein encoded by *wapl* controls heterochromatin organization and was identified as a modifier of both PEV and chromosome inheritance [2,3]. Thus, hWAPL is also expected to be involved in heterochromatin maintenance and epigenetic control.

Polycyclic aromatic hydrocarbons (PAHs) are carcinogenic and immunotoxic chemicals widely distributed in the environment [4]. 3-Methylcholanthrene (3-MC) is one of the most toxic and the best-studied compounds in the PAHs. Most of the toxic effects of PAHs are mediated by the aryl hydrocarbon receptor (AhR) [5]. When PAHs bind to the AhR, the ligated AhR translocates from the cytoplasm to the nucleus where it switches its partner molecule from heat shock protein 90 kD (Hsp90) to the aryl hydrocarbon receptor nuclear translocator (Arnt) [6]. The resulting AhR/Arnt heterodimer binds a specific DNA sequence, designated xenobiotic responsive element (XRE), in the promoter region of target genes to enhance their expression [6]. On the other hand, several studies have suggested the existence of AhR independent pathways for PAH toxicity [7,8]. In all cases, many of the putative target genes responsible for the toxicity symptoms have yet to be identified.

In the present study, we demonstrate that *hWAPL* is a target gene of 3-methylcholanthrene. The results suggest that carcinogenesis by 3-MC may involve alterations of *hWAPL* gene expression.

2. Materials and methods

2.1. Chemicals

3-Methylcholanthrene (Sigma-Aldrich Japan, Tokyo, Japan) was prepared in dimethylsulfoxide (DMSO) for cultured cells and in olive oil for treatment of mice. Aphidicolin (Wako Pure Chemical Industries, Ltd, Osaka, Japan), Nocodazole (Sigma Chemical Co., St Louis, MO) and α-naphthoflavone (Sigma) were prepared in DMSO.

2.2. Cell cultures

The human uterine cervical carcinoma-derived cell lines, SiHa, CaSki and HeLa cells, were obtained from American Type Culture Collection (ATCC), and grown in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS) (Trace Scientific Ltd, Melbourne, Australia) at 37 °C in a 5% CO_2 environment. Where indicated, SiHa cells were grown in DMEM supplemented with 10% charcoal/dextran treated FBS (CTF) (Biosource, Rockville, MD) or 0.4% (w/v) bovine serum albumin (BSA) (Trace) instead of FBS.

2.3. Immunoblot analysis

Protein samples were prepared as previously described [9]. Immunoblot analysis was performed as previously described [1].

2.4. Flow cytometric analysis

To determine cell cycle profiles, cells at different time points were harvested, washed, and fixed with a solution containing 70% ethanol and 30% PBS. After incubation overnight at 4 °C, cells were suspended in staining buffer (propidium iodide, 50 μg/ml; RNaseA, 0.1%; glucose, 1 mg/ml in PBS). Then, after incubation for 30 min at room temperature, the cells were analyzed with a FACS Vantage flow cytometer using the Cell Quest acquisition and analysis program (BD Biosciences, San Jose, CA).

2.5. Animals and treatment

C57/BL6 female mice (6 weeks old) were purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). The mice received a single intraperitoneal injection of 1 ml of olive oil containing 3-MC at a dose of 80 mg/kg of body mass. The control mice were injected with olive oil alone. Uterus samples were harvested 24 and 48 h after injection and subjected to real time PCR analysis.

2.6. RNA isolation and quantitative real time PCR

First strand cDNA synthesis was performed as described [10] using M-MLV Reverse transcriptase (Invitrogen Japan, Tokyo, Japan) with Oligo $(dT)_{17}$ (for Figs. 1–3 and 6) or Random Primers (Invitrogen) (for Figs. 4 and 5).

Real time PCR analysis for *hWAPL* and human β-*actin* mRNAs was performed as described [1]

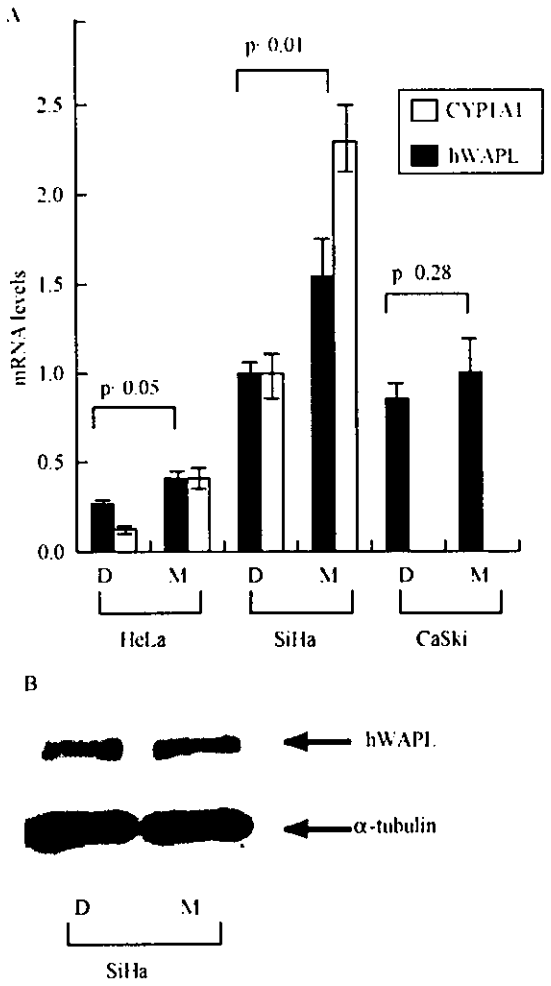

Fig. 1. Effects of 3-MC on *hWAPL* expression in the human cervical cancer-derived cell lines. D, DMSO alone; M, 3-MC. (A) HeLa, SiHa and CaSki cells were treated with 0.1% DMSO alone or 1 μM of 3-MC for 6 h. Then, the *hWAPL* and *CYP1A1* mRNA levels in the cells were evaluated by quantitative real time PCR analysis. Data were normalized to the mRNA levels of SiHa cells treated with DMSO alone that was arbitrarily set to 1 in the graphical presentation. *Bars, s.e.* (B) SiHa cells were treated with 0.1% DMSO alone or 1 μM 3MC for 6 h, and then the protein samples were prepared and subjected to western blotting analysis. α-tubulin was also shown as a loading control.

except for the 40 PCR cycles at 95 °C for 3 s and 68 °C for 30 s. Real Time PCR analysis for human *CYP1A1* mRNA and *hWAPL* hnRNA was also performed with the same PCR protocol. The nucleotide sequences of primers specific for human *CYP1A1* mRNA were previously described [11]. Primers specific for *hWAPL* hnRNA are 5′-GAGATTACACCACTGCACTCC-3′ and 5′-TTGCTCCCACTTACTATGGCC-3′. For mouse cDNAs, we used primers specific for the mouse homolog of *hWAPL* mRNA, 5′-ACCTGGTGGAGTATAGTGCCC-3′ and 5′-TGGCAGAGACACCCAAGAAGC-3′ (The nucleotide sequences were obtained from mKIAA0261 in Database), mouse β-*actin* mRNA, 5′-AGCCTTCCTTCTTGGGTATGG-3′ and 5′-CACTTGCGGTGCACGATGGAG-3′, and mouse *CYP1A1* mRNA, 5′-TTTGGTTTGGGCAAGCGA-3′ and 5′-GTCTAAGCCTGAAGATGC-3′. Reaction mixtures were denatured at 95 °C for 30 s then subjected to 40 PCR cycles at 95 °C for 3 s, 68 °C for 30 s, and 86 °C for 6 s for mouse *WAPL* mRNA, and at 95 °C for 3 s, 68 °C for 30 s, and 85 °C for 6 s for mouse β-*actin* and *CYP1A1* mRNAs, respectively. *hWAPL*, *mouse WAPL* and human and mouse *CYP1A1* mRNA levels and *hWAPL* hnRNA level were normalized to human and mouse β-*actin* signals, respectively. The absence of PCR products after the PCR on non-reverse-transcribed total RNA served as a routine check for contaminating genomic DNA. We performed the experiments to determine mRNA and hnRNA levels in triplicate.

The data were analyzed using Student's-*t* test, and $Ps < 0.05$ were considered to indicate significant differences.

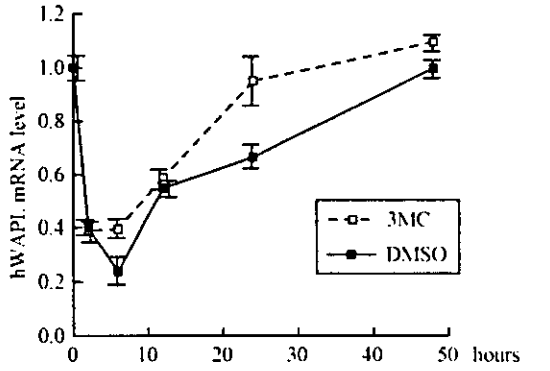

Fig. 2. Kinetics of *hWAPL* mRNA levels in SiHa cells at several time points after treatment with DMSO alone or 1 μM of 3-MC. The *hWAPL* mRNA levels in the cells were evaluated by quantitative real time PCR analysis. Data were normalized to the mRNA level at 0 h that was arbitrarily set to 1 in the graphical presentation. *Bars, s.e.*

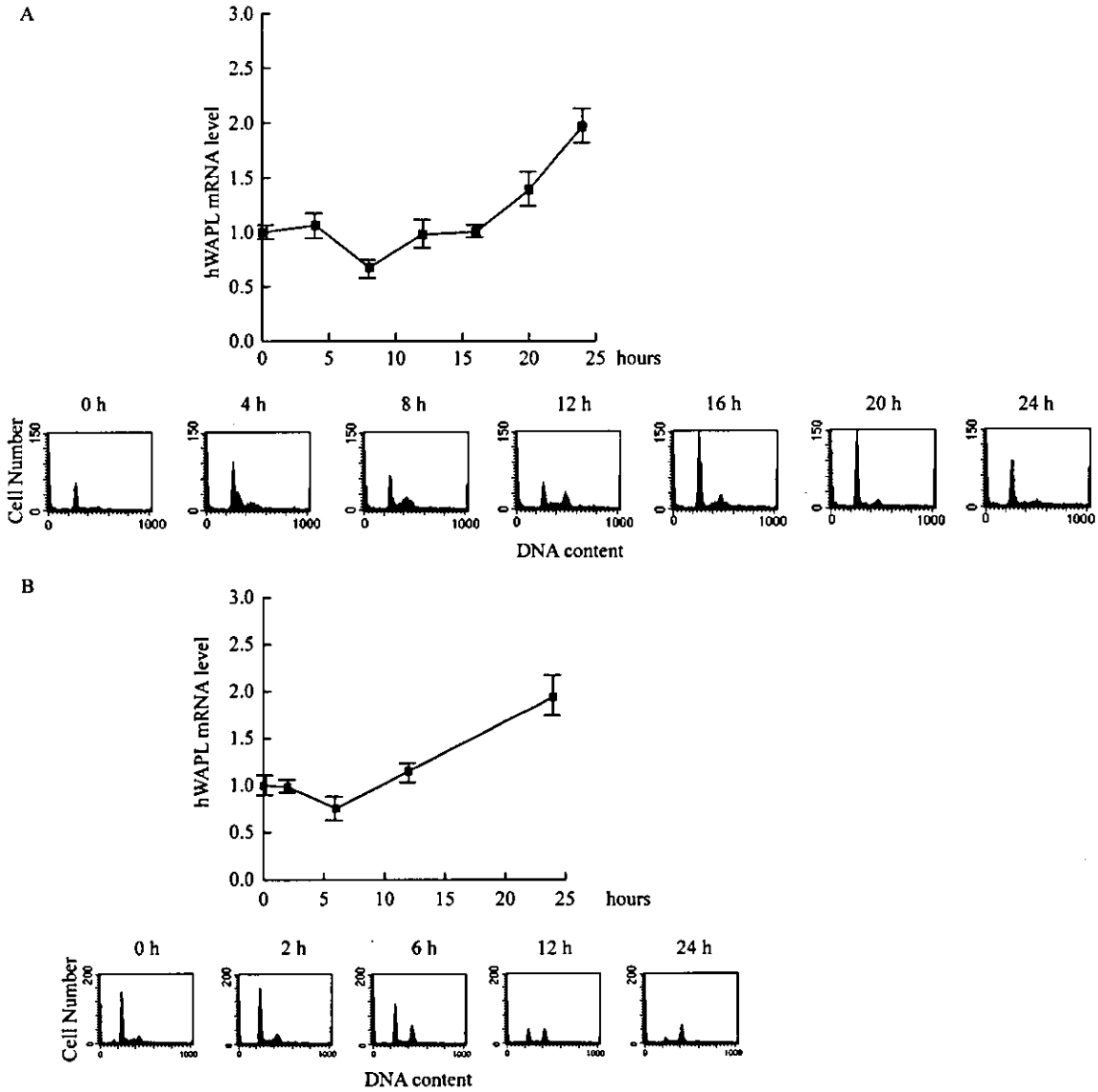

Fig. 3. Cell cycle profiles and *hWAPL* mRNA levels. *hWAPL* mRNA levels in the cells were determined by real time PCR analysis. Data were normalized to the mRNA level at 0 h that was arbitrarily set to 1 in the graphical presentation. *Bars, s.e.*. Cell cycle profiles of the cells at each time points were also confirmed by flow cytometric analysis. (A) Kinetics of *hWAPL* mRNA levels in SiHa cells at 0, 4, 8, 12, 16, 20 and 24 h after releasing from G1 arrest by 1 μg/ml aphidicolin treatment. (B) Kinetics of *hWAPL* mRNA levels in SiHa cells at 0, 2, 6, 12 and 24 h after 50 ng/ml nocodazole treatment.

3. Results and discussion

To examine whether 3-MC affects *hWAPL* expression, we treated various human uterine cervical cancer-derived cell lines with dimethylsulfoxide (DMSO) alone or 3-MC for 6 h. Then, we calculated the amounts of the *hWAPL* mRNAs in the cells by quantitative real time PCR analysis, and found that *hWAPL* mRNA levels were increased in the 3-MC-treated cells (Fig. 1A). The increases in *hWAPL* mRNA levels in SiHa cells was most remarkable among the cell lines examined. Because the *CYP1A1* gene is

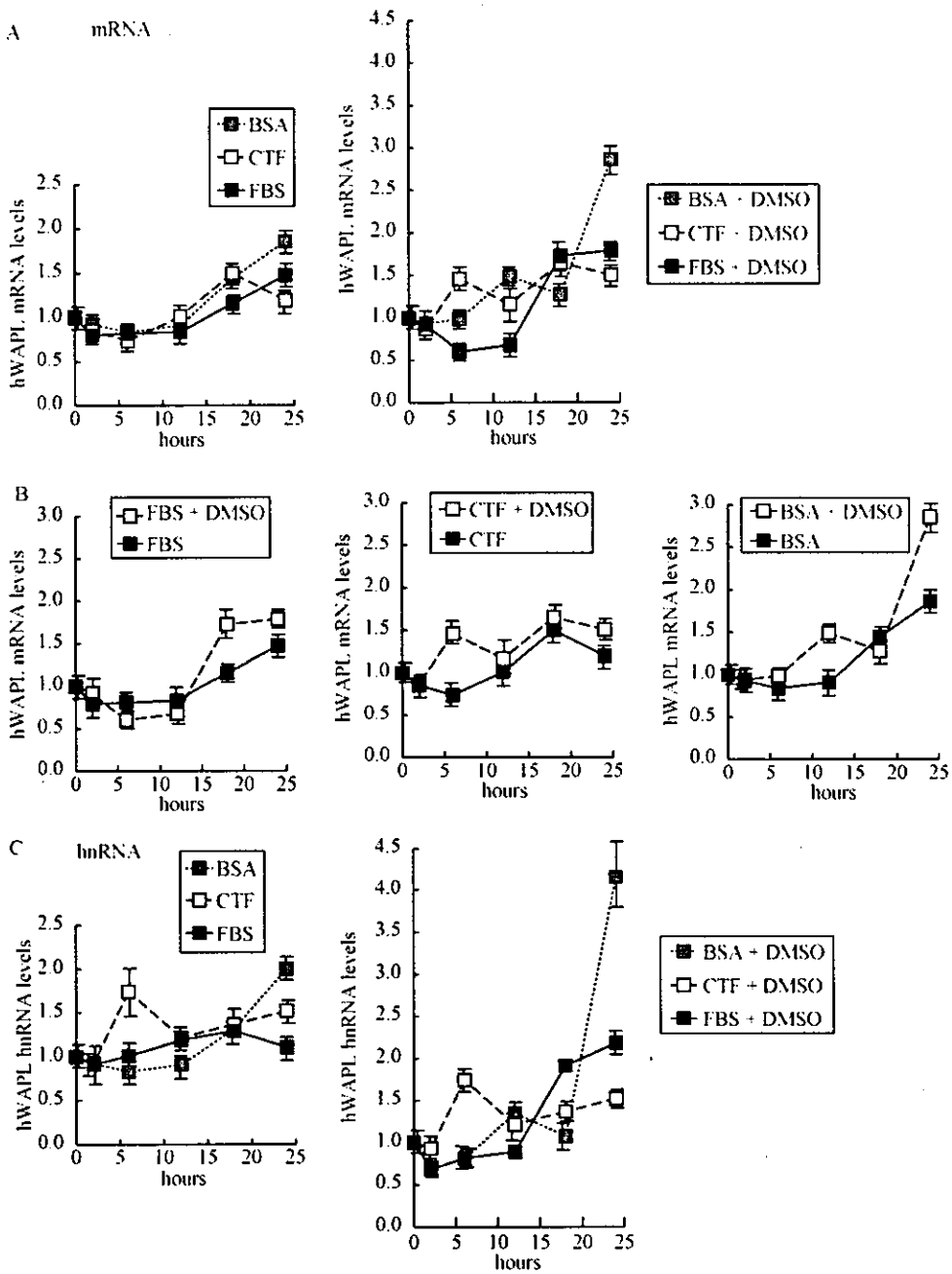

Fig. 4. Effects of FBS, CTF, BSA and DMSO on *hWAPL* mRNA and hnRNA levels in SiHa cells. *hWAPL* mRNA and hnRNA levels in SiHa cells at 0, 2, 6, 12, 18 and 24 h after replacing the growth medium to a fresh medium supplemented with FBS, CTF or BSA with or without 0.1% DMSO were deteremined by real time PCR analysis. Data were normalized to the mRNA and hnRNA level at 0 h that was arbitrarily set to 1 in the graphical presentation. *Bars, s.e.* (A) Kinetics of the *hWAPL* mRNA levels in the cells grown in the growth medium supplemented as indicated. (B) Graphical representation of the effects of DMSO on the kinetics of *hWAPL* mRNA levels in the cells grown in the growth medium supplemented with FBS, CTF or BSA. (C) Kinetics of the *hWAPL* hnRNA levels in the cells grown in the growth medium supplemented as indicated.

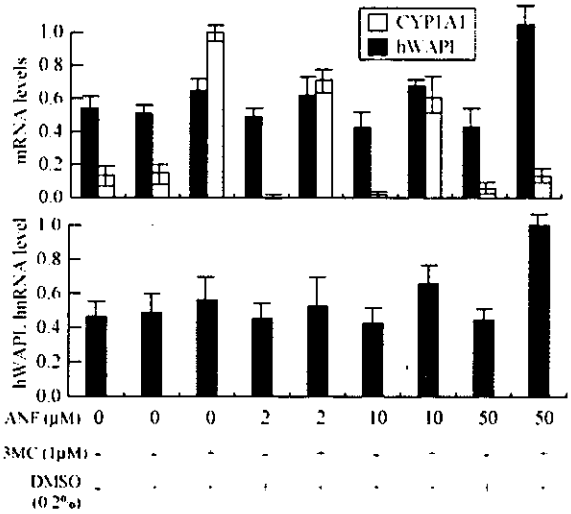

Fig. 5. Effects of AhR inhibition by α-naphthoflavone on *hWAPL* mRNA and hnRNA levels in SiHa cells treated with 3-MC. SiHa cells were treated with 0.2% DMSO alone or 1 μM 3-MC with 0. 2, 10 or 50 μM ANF for 6 h. Then, the *hWAPL* mRNA and hnRNA levels and the *CYP1A1* mRNA levels were deteremined by real time PCR analysis. SiHa cells grown for 6 h in a normal fresh medium without chemicals were also analyzed as a normal control. Data were normalized to the maximaum mRNA and hnRNA levels that were arbitrarily set to 1 in the graphical presentation. *Bars, s.e.*

a well-known target of 3-MC [12,13], we also calculated *CYP1A1* mRNA levels in the three cell lines to confirm the effects of 3-MC on the cells (Fig. 1A). We found that *CYP1A1* mRNA levels in SiHa cells were highest and increased most remarkable. *CYP1A1* mRNA in CaSki cells was not detected in our experiments. We also observed that hWAPL protein level was increased in the 3-MC-treated SiHa cells (Fig. 1B).

We next examined the effects of 3-MC on *hWAPL* expression in SiHa cells at several time points after 3-MC treatment (Fig. 2). The 3-MC-treated cells showed higher levels of *hWAPL* mRNA than the control cells at all time points examined. Interestingly, the *hWAPL* mRNA levels decreased first 6 h and then increased after changing the medium to a fresh medium containing DMSO with or without 3-MC as seen in Fig. 2.

These results prompted us to investigate whether the *hWAPL* expression is related to the cell cycle. First, to synchronize cell cycle progression, we treated SiHa cells with aphidicolin, an inhibitor of DNA synthesis, for 12 h to induce G1-phase arrest. We then released the cells from G1 arrest by changing the culture medium to a fresh growth medium. The synchronized cells were harvested every 4 h for 24 h after release from aphidicolin, and the *hWAPL* mRNA levels were calculated by quantitative real time PCR analysis (Fig. 3A). As seen in Fig. 3A, *hWAPL* mRNA initially decreased and then increased over time. Flow cytometric analysis confirmed the cell cycle phase of the cells at each time point (Fig. 3A). From these results, *hWAPL* mRNA level seemed to fluctuate in accordance with cell cycle profile. However, the levels of *hWAPL* mRNA in the cells treated with nocodazole, an inhibitor of spindle assembly, fluctuated in a similar manner to the aphidicolin-synchronized cells (Fig. 3B). Thus, amounts of *hWAPL* mRNAs are likely to have no relation to the cell cycle profiles. Recently, Guigal et al. demonstrated that FBS induces transcription of the *CYP1A1* gene. Therefore, we suspected that the fluctuation of *hWAPL* mRNA levels might be associated with the culture medium change.

To investigate the effects of components in FBS on the fluctuation of *hWAPL* mRNA levels, we examined the *hWAPL* mRNA levels in SiHa cells after changing growth medium to a fresh medium supplemented with charcoal/dextran treated FBS (CTF) or BSA instead of FBS. The fluctuations of the *hWAPL* mRNA levels showed similar trends among the cells grown with FBS, CTF and BSA (Fig. 4A; left panel). However, all cells examined in Figs. 1–3 were grown in the medium containing DMSO. Thus, we also tested the effects of DMSO with FBS, CTF or BSA at the same time. Interestingly, fluctuations of the *hWAPL* mRNA levels in SiHa cells treated with 0.1% DMSO showed different trends among FBS, CTF and BSA (Fig. 4A; right panel), and the *hWAPL* mRNA levels in the DMSO-treated cells fluctuated more drastically than that in the cells grown without DMSO (Fig. 4B). Especially, remarkable decrease of *hWAPL* mRNA levels for first 6 h after the medium change was distinctive for the growth medium supplemented with FBS and DMSO. These results suggest that DMSO and some constituents of FBS affect *hWAPL* mRNA accumulation synergistically.

mRNA levels do not always reflect on the transcription activity of genes. To investigate the kinetics of the promoter activities of the *hWAPL* gene in the cells, we evaluated the levels of *hWAPL* heterogeneous nuclear RNA (hnRNA), the unprocessed precursor of the mature and functional mRNA,

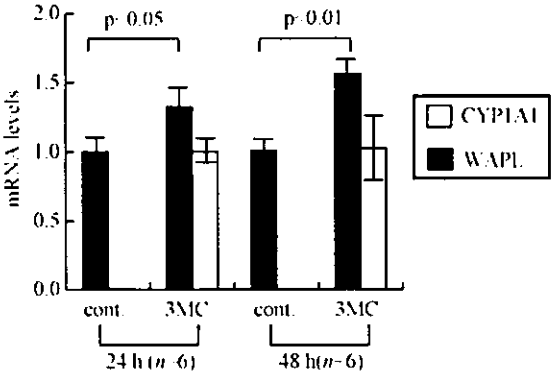

Fig. 6. Effects of 3-MC on *WAPL* mRNA levels in mouse uteri. The mice received a single intraperitoneal injection of 1 ml of olive oil containing 3-MC (3-MC) or olive oil only (cont.), and *WAPL* mRNA levels in the uteri at 24 and 48 h after injection were determined with quantitative real time PCR analysis. *CYP1A1* mRNA levels were also deteremined to confirm the effects of 3-MC on mouse uteri. The data represent the means of multiple samples normalized to the mean values of the *hWAPL* mRNA levels in the cont. 24 h samples and *CYP1A1* mRNA levels in the 3-MC 24 h samples, respectively, that were arbitrarily set to 1 in the graphical presentation. *Bars, s.e.*

by real time PCR using intron-specific primers for *hWAPL* searched in Ensembl Genome Browser (http://www.ensembl.org/). Levels of hnRNA have been proposed as a surrogate for nuclear run-on assays to determine gene transcription rates [14,15]. Although the *hWAPL* hnRNA levels fluctuated in somewhat different manner to the mRNA levels in the cells grown without DMSO, the *hWAPL* hnRNA levels in the DMSO-treated cells fluctuated in similar manner to the mRNA levels (Fig. 4C; compare with Fig. 4A). These results suggest that DMSO and some components of FBS affect transcriptional activity of the *hWAPL* gene. Increase of *hWAPL* transcription levels at 24 h in common with the cells under various conditions may be caused by the accumulation of wastes in their growth medium.

3-MC is known to be an agonist of AhR [16]. Thus, to investigate whether AhR is related to *hWAPL* transcription activation, we examined the effects of α-naphthoflavone (ANF), an AhR antagonist [17], at a dose of 2, 10 and 50 μM on *hWAPL* mRNA and hnRNA levels in 3-MC-treated SiHa cells by quantitative real time PCR analysis (Fig. 5). We also evaluated *CYP1A1* mRNA levels for monitoring the inhibitory effects on AhR functions by ANF, and found that 50 μM of ANF strongly inhibited AhR functions (Fig. 5; upper panel). Interestingly, increase of *hWAPL* mRNA levels by 3-MC was more remarkable in AhR-inhibited cells rather than that in AhR-functioning normal cells. Induction of *hWAPL* hnRNA levels showed similar manner to the *hWAPL* mRNA (Fig. 5; lower panel). From these results, we hypothesized that AhR was involved in the transcriptional regulation of *hWAPL*, but there are complex mechanisms for the transcriptional regulation of *hWAPL*. We did not find XRE motif in 5000 bp of 5′-upstream sequence of the *hWAPL* gene using MOTIF Sequence Motif Search (http://motif.genome.jp/) at the cut off score 85. Thus, although further investigation is required, we suppose that *hWAPL* is not a direct target of 3-MC but a down stream molecule of a 3-MC-targeted molecule.

Finally, we examined whether the mRNA level of a mouse homolog of *hWAPL* is increased by 3-MC in mouse uterus. Twenty-four and 48 h after the injection of 3-MC into the abdominal cavities of C57/BL6 female mice, we harvested the uteri and analyzed the *WAPL* mRNA levels by quantitative real time PCR analysis. The *CYP1A1* mRNA levels were also analyzed to confirm the 3-MC effects on the uteri. The uteri exhibited increases in *WAPL* mRNA levels compared with that of control mice (Fig. 6). These data suggest that 3-MC exposure affects *WAPL* expression in uterus.

Our recent data demonstrated that the unscheduled increase of *hWAPL* expression in human uterine cervix is associated with cervical cancer [1]. In Addition, previous studies demonstrated that 3-MC induces carcinogenesis in mouse uterine cervix [18, 19]. Thus, although the *hWAPL* induction by 3-MC was weak in our experiments, our results suggest that the promotion of carcinogenesis by 3-MC in uterus is likely to involve the *hWAPL* oncogene.

Acknowledgements

This work was supported by a Grant-in-Aid for scientific research on Priority Area (C) from the Ministry of Education, Science, Sports and Culture, and a grant from Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation.

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