Polycomb group (PcG) proteins are evolutionary conserved complexes from flies to mammal that regulate body segmentation through the modulation of higher order chromatin structure [8]. In mammals, PcG proteins form at least two distinct repressive protein complexes, PRC1 and PRC2, that catalyze histone modifications such as ubiquitylation of H2A at K119 and methylation of histone H3 at K27, respectively [9]. PcG complexes are critical components of the molecular memory that maintain gene expression patterns beyond cell proliferation and PcG-mediated gene silencing is essential for maintaining stemness in various types of stem cells through suppressing expression of genes determining differentiation phenotypes [10,11]. Notably, mice lacking Scmh1, cbx2, and those lacking both Ring1B and Ring1A demonstrated severe defects in testis development, suggesting roles of PRC1 in spermatogenesis [12–15].

Spermatogenesis is a complex and coordinated process for cellular differentiation and is essential for sexual reproduction [16]. The development of male germ cell is performed through intricate and highly ordered sequential steps including meiosis, genetic recombination, haploid gene expression, acrosome and flagellum formation, chromatin remodeling and condensation [17]. These processes should therefore require a precise and stringently controlled program for chromatin reorganization. Like any other differentiating cells, the characteristics of the individual cell types that comprise the testis are dependent on differential gene expression including testis-specific genes or isoforms, which can be successfully achieved through complex networks of epigenetic regulation and interactions between transcription factors [18].

In order to address the physiological function of MPP8 during spermatogenesis, we performed immunohistochemical staining in the testis and found that it localized predominantly in spermatocytes and modestly in spermatogonia, while weak or very faint staining could be observed in spermatids and mature sperm. Importantly, MPP8 physically interacted with Ring1B and Bmi1, components of PRC1 and knockdown analysis in HeLa cells revealed that MPP8 is required for silencing a group of genes that are normally expressed in spermatogonia and spermatids. Depletion of MPP8 in ES cells specifically induced expression of genes involved in mesoderm differentiation, such as Cdx2 and Brachyury. Thus, our results suggest a previously unidentified role for MPP8 in testis development and spermatogenesis by regulation of testis-specific gene expression, possibly through modulating PRC1 function.

2. Materials and methods

2.1. Yeast two-hybrid screening

The *pGBKT7-MPP8* plasmid was generated by insertion of the full-length human MPP8-encoding sequence. *pGBKT7-MPP8* was transformed into the yeast strain AH101 and mated with yeast Y187 pretransformed with a HeLa cell cDNA library (BD biosciences).

2.2. Immunohistochemistry of rat testis sections

The testes, heart, brain, liver, small intestine, ovary, and endometrium of F344 wild type rat were removed and submerged into 10% (v/v) neutral buffered formalin for 6 h, embedded in paraffin and sectioned at a thickness of 3 μm.

Immunohistochemical staining was applied on paraffin-embedded sections (3 mm thick) of testes and multiple organs of F344 rats. Immunostaining was performed on a VENTANA DISCOVERY HX auto-immunostainer (Roche, Mannheim, Germany). Antigen retrieval was carried out with CC1 buffer (Roche) for 60 min at 100 °C. Sections were incubated for 60 min with antibodies specific for primary antibodies and sequentially with secondary antibodies and avidin–biotin complex (Vectastatin Elite ABC kit; Vector Laboratory, Burlingame, CA), then binding sites were visualized with diamino-benzidine (DAB; Sigma). Sections were lightly counterstained with hematoxylin to facilitate microscopic analysis. Microscope images were obtained using Olympus AX70 (Olympus, Tokyo, Japan).

2.3. Cell culture

HeLa cells were cultured in DMEM supplemented with 10% FBS. E14 mES cells were cultured on 0.1% gelatin-coated plates in DMEM supplemented with 15% FBS, 1 mM Sodium Pyruvate (Gibco), 1 × MEM non-essential amino acid (Gibco) and 10^3units ESGRO mouse LIF medium supplement (Millipore). All cells were cultured at 37 °C under 5% CO_2.

2.4. Lentiviral transduction

Lentivirus expressing the MPP8 shRNA (GCAACAGATGCAATTCCAAGT) was generated by the co-transfection of 293T cells with pCMV-VSV-G-RSV-RevB (a gift from H. Miyoshi), pCAG-HIVgp (also a gift from H. Miyoshi), and the respective CSIV-TRE-RfA-UbC-KT using the calcium phosphate co-precipitation method. Cells infected with viruses were treated with 10 μg/ml Blasticidin for 3 days. To knockdown of MPP8, Doxycycline (Sigma–Aldrich) was added to the medium at the concentration of 1 μg/ml.

2.5. Nuclear protein extraction, immunoprecipitation and immunoblotting

Nuclear protein extraction for immunoprecipitation, HeLa cells were grown to ≤80% confluence, trypsinized, and centrifuged (200× *g* for 3 min at room temperature), then washed in ice cold PBS. Protein was prepared as described previously [19]. Immunoprecipitation, nuclear extracts were diluted with buffer D (20 mM Hepes-KOH, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol) and incubated with 1 μg of primary antibodies at 4 °C for overnight and incubated with 10 μl of protein A-agarose (Roche) at 4 °C for 1 h Beads were thoroughly washed three times with buffer D and resuspended in 1 × SDS sample buffer and boiled 100 °C for 5 min. Sample was analyzed by immunoblotting.

2.6. Antibodies

The antibodies used in this study were MPP8 (16796-1-AP, proteintech group), Bmi1 (D20B7, Cell signaling), Flag (F3165, sigma), Ring1B (for immunoblotting, 39663, active motif and for immunoprecipitation, ab101273, abcam).

2.7. Microarray experiment

HeLa cells infected shMPP8 were cultured with/without Doxycycline for 3 days and total RNA was prepared using ISOGENII (Nippongene). RNA hybridization, wash and analysis were performed using SurePrint G3 Human GE 8×60k Ver2.0 (Agilent Technologies), GeneSpring GX Ver12.6.0 (Agilent Technologies). Microarray expression profiling was performed by Oncomics (Nagoya, Japan).

2.8. Data analysis

Tests for gene ontology (GO) and tissue enrichment patterns were performed using DAVID (the Database for Annotation,

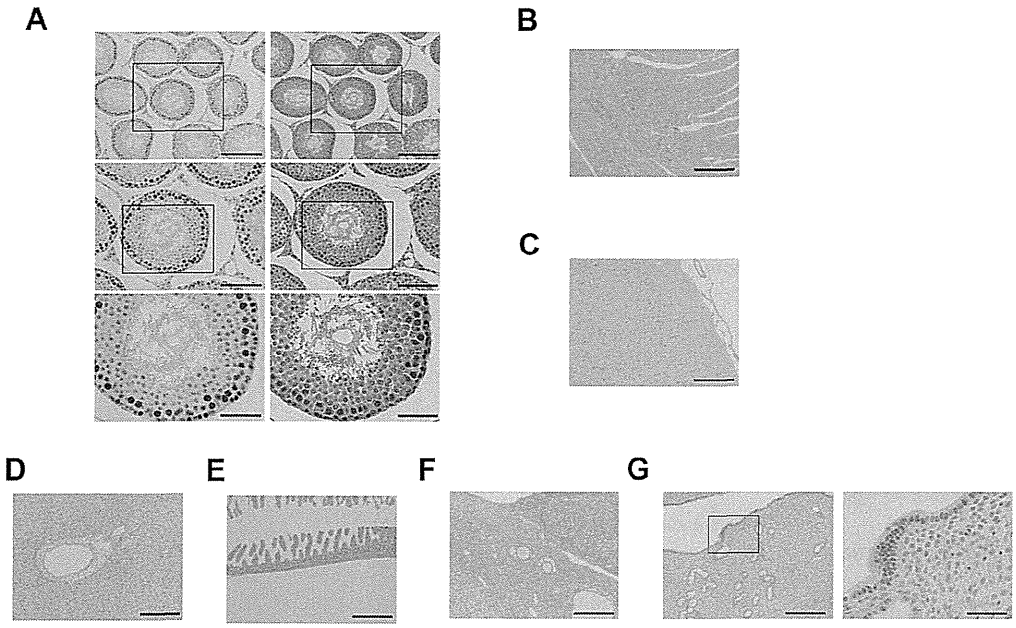

Fig. 1. MPP8 is predominantly expressed in spermatocytes and modestly in spermatogonia.
Immunohistochemical staining of paraffin-embedded rat testis (A), heart (B), brain (C), liver (D), small intestine (E), ovary (F) and endometrium (G) using Anti-MPP8. HE staining was also shown in (A). Bar = 200 μm in (A) top panel, (B)–(F) and (G) left panel, 100 μm in (A) middle panel, 50 μm in (A) bottom panel and (G) right panel.

Visualization and Integrated Discovery; http://www.david.abcc.ncifcrf.gov/) web resources. ChIP-seq data for RNA polymerase II (Pol-II), H3K27me3, H3K4me3 and H3K9me3 enrichment in K562 cells were obtained from the publicly available GEO database (gene expression omnibus, http://www.ncbi.nlm.nih.gov/geo/). These datasets were deposited by Bernstein et al. (MGH, Harvard) and the accession number for each ChIP-seq experiment is shown in Fig. 3C. ChIP-seq data were visualized with the IGV (integrative genomics viewer) browser distributed by Broad Institute (http://www.broadinstitute.org/igv/). RNA-seq datasets for testicular cells (spermatogonia: Sg, spermatocytes: Sc; spermatids: Std; spermatozoa: Sz) deposited by Soumillon et al. [20] were retrieved from the GEO database (series accession number GSE43717). University of Toronto BAR (Bio-Analytic Resource, http://www. bar.utoronto.ca/) tools were used for heatmap analysis.

2.9. RT-qPCR assay

Tet-inducible shRNA transfected E14 mES cells were cultured with 1 μg/ml doxycycline with presence of LIF medium for 7 days. RNA was extracted from E14 mES cells with IsogenII (Nippon Gene). 5 μg of total RNA were converted to cDNA using ReverTra Ace qPCR RT kit (TOYOBO). cDNA levels were assayed by real-time PCR with FastStart Universal SYBR Green Master (Roche) and analyzed on real time PCR ABI7500fast (Applied Biosystems). PCR was performed using following primers:

Cdx2 F (5′-AAGACAAATACCGGGTGGTG-3′),
Cdx2 R (5′-CCAGCTCACTTTTCCTCCTG-3′),
Brachyury F (5′-TCCCGAGACCCAGTTCATAG-3′),
Brachyury R (5′-TTCTTTGGCATCAAGGAAGG-3′),
Arbp F (5′-CAAAGCTGAAGCAAAGGAAGAG-3′), and
Arbp R (5′-AATTAAGCAGGCTGACTTGGTTG-3′).

The expression of individual genes was normalized to the level of Arbp.

3. Results and discussion

3.1. MPP8 is predominantly expressed in spermatocytes and modestly in spermatogonia

Immunohistochemical analysis in rat testis using specific antibodies to MPP8 revealed robust staining in spermatocytes and spermatogonia, while the staining faint in spermatid, and sperm (Fig. 1A). Tissue-specific immunohistochemical analyses demonstrating hardly detectable levels of MPP8 in heart (Fig. 1B), brain (Fig. 1C), liver (Fig. 1D), small intestine (Fig. 1E), ovary (Fig. 1F), and endometrium (Fig. 1G). These results were consistent with our observations from tissue-specific quantitative real-time PCR analyses which showed that MPP8 is mostly expressed in testis [7]. Taken together, these results suggested that MPP8 is predominantly and modestly expressed in spermatocytes and spermatogonia

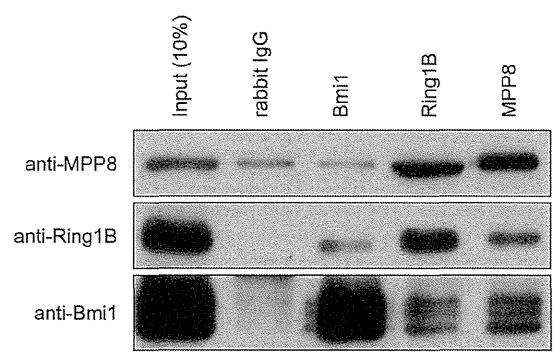

Fig. 2. MPP8 physically interacts with PRC1 components, Ring1B and Bmi1. Nuclear extracts prepared from HeLa cells were immunoprecipitated with the indicated antibodies. The resultant immunoprecipitates were subjected to immunoblotting using the indicate antibodies.

A

Term	Count	%	P-Value	Fold Enrichment	FDR
Brain cortex	6	1.5	6.20E-02	2.8	5.40E+01
Keratinocyte	6	1.5	6.90E-02	2.7	5.80E+01
Testis	84	20.5	8.30E-02	1.2	6.50E+01
Hippocampus	13	3.2	9.10E-02	1.7	6.80E+01

Tissue-wise enrichment of upregulated genes upon MPP8 knockdown

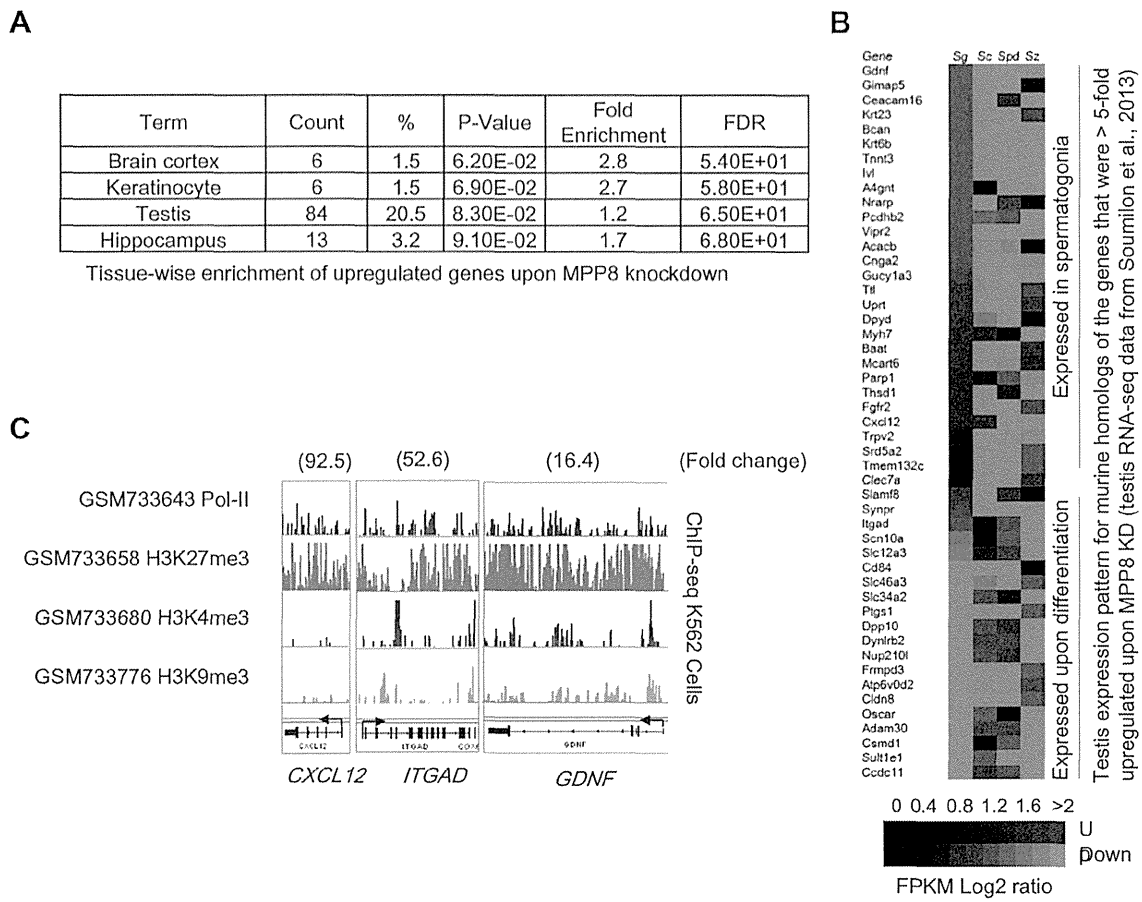

Fig. 3. MPP8 represses genes that are transcribed in spermatogonia, spermatids and spermatozoa. (A) Tissue enrichment patterns for upregulated genes were analyzed. The table shows the list of enriched tissues, number (count) and percentage of genes (%), p-value, fold enrichment and FDR. (B) RNA-seq (FPKM) expression patterns of upregulated (>5-fold) genes. The types of testicular cells are delineated as Sg (spermatogonia), Sc (spermatocytes), Std (spermatids) and Sz (spermatozoa). Fold expression levels (compared to the median, Log2 values) are represented in a heatmap. The color scale of the heatmap is given at the bottom. (C) ChIP-seq profiles for RNA polymerase II (Pol-II), H3K27me3, H3K4me3, and H3K9me3 are exhibited for representative genes that were found to be upregulated upon MPP8 KD. The GEO accession number of each dataset is shown at the left. Gene names are given at the bottom of each panel and the fold upregulation (upon MPP8 KD) are shown at the top.

respectively and therefore could be involved in regulation of spermatogenesis.

3.2. MPP8 physically interacts with PRC1 components

In order to determine the physiological relevance of MPP8 in spermatogenesis, we performed yeast two-hybrid screening using MPP8 as a bait. Of a total of 5×10^6 transformants from a HeLa cell cDNA library, 27 positive colonies were confirmed to be lacZ-positive. They contained overlapping several cDNAs, such as genes derived from MBD4, WBP5, and Ring1B (Table 1). Even among them, we will focus on Ring1B, a component of PRC1 complexes, because PRC1 was reported to express most highly in testis and to be involved in spermatogenesis [13]. Physical interaction of MPP8 with Ring1B was confirmed by IP-western blotting analysis. As shown in Fig. 2, Ring1B was readily detected in MPP8 immunoprecipitates from HeLa cells. Interestingly, Bmi1, another component of PRC1, was also detectable in MPP8 immunoprecipitates, suggesting that MPP8 forms a complex with PRC1 complex. Consistent with this, MPP8 was also detected in Ring1B immunoprecipitates. Taken together, these results indicate that MPP8 appears to form a stable complex with PRC1.

Table 1
The isolated genes by two-hybrid screening using human MPP8 as bait.

Gene	RefSeq ID	Function	Clone number
WBP5	NM_001006612.1	WW domain binding	4
MBD4	NM_001276270.1	DNA repair and DNA demethylation	3
Ring1B	NM_007212.3	Transcriptional regulation, ubiquitin ligase	3
FAM968		Unknown	2
v-myc		Transcriptional regulation	2
c-myc	NM_002467.4	Transcriptional regulation	1
CCDC34	NM_030771.1	Unknown	1
CHRAC1	NM_017444.5	Histone-fold protein	1
GRAMD4	NM_015124.3	Mitochondrial effector of E2F1-induced apoptosis	1
HINT2	NM_032593.2	Nucleotide hydrolases	1
ISCA2	NM_001272007.1	Metal ion binding	1
MBD1	NM_001204136.1	Transcriptional regulation	1
NSD1	NM_022455.4	H3-K36-HMTase, H4-K20-HMTase	1
SAP30BP	NM_001301839.1	Positive regulation of cell death	1
SKP1	NM_006930.3	The component of SCF complexes	1
SPCS2	NM_014752.2	Peptidase activity	1
USP8	NM_001128610.2	Ubiquitin-specific protease activity	1
WWC1	NM_001161661.1	Cytoplasmic phosphoprotein that interacts with PRKC-zeta and dynein light chain-1	1

3.3. Depletion of MPP8 in HeLa cells resulted in transcriptional activation of genes that are normally expressed in spermatogonia

To elucidate the function of MPP8 in global gene regulation, we performed microarray analyses in HeLa cells in which the MPP8 gene was knocked down by shRNAs. Microarray results revealed that approximately 800 and 500 genes were upregulated or downregulated (by > 2-fold), respectively, upon MPP8 knockdown (KD). To gain insight into the role of the genes that were upregulated after MPP8 KD, we performed functional annotation of the upregulated genes by using the DAVID (Visualization and Integrated Discovery) tools and found that many of these genes (84 annotations) associated with the "testis" tissue category, supporting our aforementioned findings that MPP8 could have a role in testis (Fig. 3A).

We went on to explore the link between MPP8 and testis-specific gene expression by analyzing publicly available datasets (#GSE43717) [20] for gene expression level in various testicular cells such as spermatogonia (Sg), spermatocytes (Sc), spermatids (Std) and spermatozoa (Sz) measured by deep sequencing (RNA-seq). We isolated the genes that were upregulated by 5-fold upon MPP8 KD in HeLa cells and extracted their murine counterparts by homology search (for example the murine Parp1 is a homolog of human PARP1). We then examined the relative expression level of these genes in Sg, Sc, Std and Sz cells using the RNA-seq data. The results are shown in a heatmap in Fig. 4B. Interestingly, we observed that the genes that were upregulated by MPP8 KD, were mainly associated with non-spermatocyte cells such as spermatogonia (Sg), spermatids (Std) and spermatozoa (Sz). For example, the Gdnf (Glial cell-derived neurotrophic factor) gene is normally expressed predominantly in the spermatogonia but not in other cell types in the testis. Similarly, the Thsd1 (thrombospondin, type I, domain containing 1) gene is strongly expressed in the spermatids (Std), while factors such as Ptgs1 (prostaglandin-endoperoxide synthase 1) exhibits a spermatozoa specific transcription pattern. These findings implicate that MPP8 might have a function to silence these genes in spermatocytes, presumably by cooperating with other factors. Intriguingly, an opposite pattern was also observed for some genes such as Sult1e1 (sulfotransferase family 1E, estrogen-preferring, member 1) and Parp1 (poly (ADP-ribose) polymerase 1), which showed enrichment in spermatocytes. It is conceivable that some of the spermatocyte expressed genes are maintained in a moderate transcriptional state under physiological conditions by MPP8 mediated repression. In the absence of MPP8, these genes were therefore more strongly expressed.

Further, to understand the link between PRC1 and MPP8, we analyzed the publicly available ChIP-seq datasets for H3K27me3, H3K4me3, H3K9me3 and RNA Pol-II deposition in the human K562 cell line. In general, we observed frequent enrichment of H3K27me3 in the genes that were upregulated upon MPP8 KD such as CXCL12, ITGAD and GDNF (Fig. 3C, fold upregulation upon MPP8 KD is shown at the top of each panel). Importantly, we did not observe a strong enrichment of H3K9me3 marks in these loci. In mammals, H3K27me3 modification is mediated by the PcG proteins [21]. Association of H3K27me3 marks with the gene loci that are derepressed upon MPP8 knockdown could be therefore linked by a putative PRC1-dependent mechanism. Our gene ontology tests and tissue enrichment examinations have indicated that MPP8 could have a role for silencing differentiation associated factors (e.g. neural genes, and genes that are expressed in differentiated spermatid cells). Polycomb factors are well-known as evolutionarily conserved modules for silencing differentiation associated genes among species [21]. We analyzed publicly available dataset of Ring1B bound regions and found that Ring1B associated with 70 genes that were up-regulated upon MPP8 knockdown, such as Gdnf and Gimap5 (series accession number GSE41316) [22]. Using a database of Ring1B knockout gene expression profile (series accession number GSE10476) [23], we also identified 9 genes, such as HMGA2 and NT5E, which were up-regulated upon MPP8 knockdown as well as Ring1B knockout cells. Taken together, these

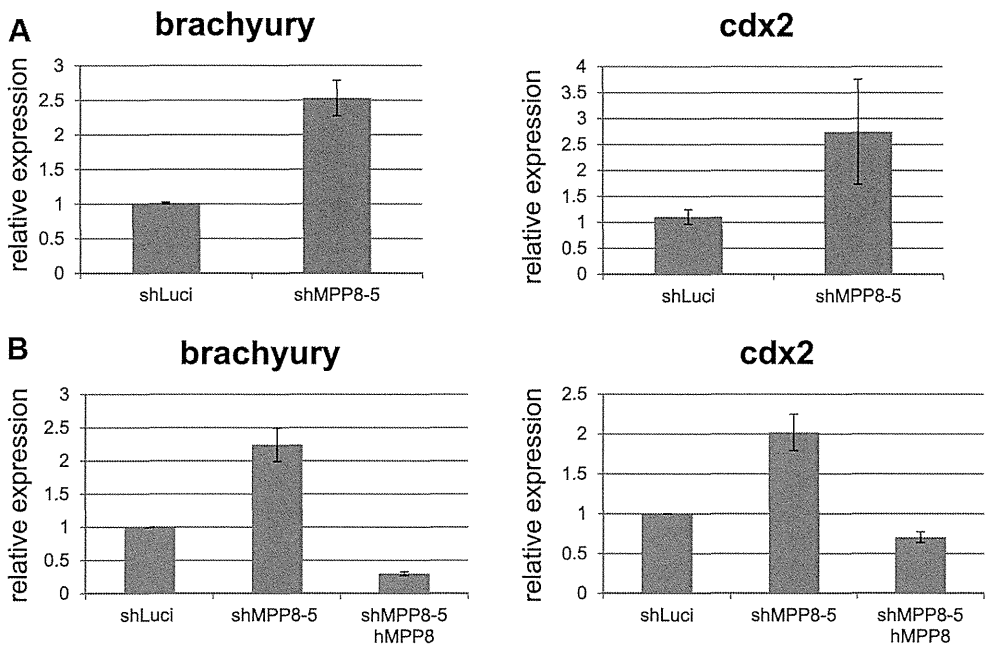

Fig. 4. Depletion of MPP8 in ES cells specifically induce the expression of mesoderm markers. (A) Mouse ES cells were infected with Tet-on inducible lentiviruses expressing shControl or shMPP8. Infected cells were cultured with Dox for 7 days in the presence of LIF. (B) Mouse ES cells expressing Tet-on inducible shControl, shMPP8 and shMPP8 and shRNA resistant human MPP8 were cultured in the presence of Dox for 7 days. Total RNAs prepared from cells from (A) and (B) were subjected to quantitative real-time RT-PCR using a set of primers within the indicated marker genes involved in pluripotency and those involved in germ-layer differentiation.

data suggests a putative association between MPP8 and the PRC1 complex.

3.4. Depletion of MPP8 in ES cells resulted in transcriptional activation of genes involved in mesoderm induction

Given that PRC1 function is essential for maintenance of pluripotency in ES cells, we speculated that it is also the case with MPP8. Thus, we examined whether depletion of MPP8 in ES cells could affect the expression of marker genes involved in germ-layer differentiation. When MPP8 was depleted from ES cells in the presence of LIF, quantitative PCR analysis revealed the specific and significant induction of mesoderm markers, such as Brachyury and Cdx2 (Fig. 4A). Importantly, increased expressions of mesoderm markers were effectively suppressed when wild-type MPP8 was ectopically expressed in endogenous MPP8-depleted ES cells (Fig. 4B), confirming the specificity of its repressive effect on the expression of mesoderm markers. Since genital organs including testis as well as hematopoietic organs and nervous systems, whose development was regulated by PRC1 complexes, were derived from mesoderm, MPP8 appears to be involved in mesoderm induction, testis development and spermatogenesis through suppression of genes specifically involved in these processes, possibly under the collaboration with PRC1 complexes.

Conflict of interest

None.

Acknowledgments

We thank Ms. Hiromi Matsuo and Dr. Chisato Yamada for technical assistance, Dr. Makoto Nishigaki and Takahisa Hirokawa for critical discussions, Dr. Hiroyuki Miyoshi for lentivirus vectors and Dr. Naohito Nozaki for antibodies. This work was supported by the NEXT program and the Naito Foundation awarded to M.S.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.122.

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RESEARCH ARTICLE

Genotyping Analysis for the 46 C/T Polymorphism of Coagulation Factor XII and the Involvement of Factor XII Activity in Patients with Recurrent Pregnancy Loss

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Citation: Asano E, Ebara T, Yamada-Namikawa C, Kitaori T, Suzumori N, et al. (2014) Genotyping Analysis for the 46 C/T Polymorphism of Coagulation Factor XII and the Involvement of Factor XII Activity in Patients with Recurrent Pregnancy Loss. PLoS ONE 9(12): e114452. doi:10.1371/journal.pone.0114452

Editor: Golo Ahlenstiel, University of Sydney, Australia

Received: September 17, 2014

Accepted: November 6, 2014

Published: December 9, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

Funding: This work was supported by a Grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, ID 24592472, (http://www.mext.go.jp/a_menu/shinkou/hojyo/main5_a5.htm). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Background: Established causes of recurrent pregnancy loss (RPL) include antiphospholipid syndrome, uterine anomalies, parental chromosomal abnormalities, particularly translocations and abnormal embryonic karyotype. A systematic review concluded that coagulation factor XII (FXII) deficiency was associated with RPL. However, it could not be established whether the 46 C/T SNP of FXII or low activity of FXII was a risk factor for RPL, because of the small sample size.

Methods and Findings: We conducted a cross-sectional and cohort study in 279 patients with two or more unexplained consecutive pregnancy losses and 100 fertile women. The association between the lupus anticoagulant (LA) activity and FXII activity was examined. The frequency of the CC, CT and TT genotypes and the FXII activity were also compared between the patients and controls. Subsequent miscarriage rates among the CC, CT, TT genotypes and according to the FXII activity was examined. LA was associated with reduced FXII activity. The CT, but not the TT, genotype was confirmed to be a risk factor for RPL in the cross-sectional study using multivariate logistic regression analysis (OR, 2.8; 95% CI, 1.37–5.85). The plasma FXII activity in the patients was similar to that in the controls. Neither low FXII activity nor the CT genotype predicted the subsequent pregnancy outcome in the cohort study. On the other hand, and intermediate FXII activity level of 85–101% was predictive of subsequent miscarriage.

Conclusions: Low FXII activity was not associated with RPL. The FXII gene was found to be one of the significant susceptibility genes for RPL, similar to the FV Leiden mutation. However, the clinical influence of the CT genotype might be relatively small, because the presence/absence of this genotype did not have any predictive value for the subsequent pregnancy outcome. This was the first study indicating the influence of *FXII 46C/T* on further pregnancy outcomes.

Introduction

Recurrent miscarriage (RM) is classically defined as three or more consecutive miscarriage [1]. However, many researchers have now revised the definition to two or more pregnancy losses, namely recurrent pregnancy loss (RPL), because of the recent increase in the prevalence of childless couples. The estimated incidence of RM and RPL are 1% and 5%, respectively [1]. Established causes of RPL include antiphospholipid syndrome (APS), uterine anomalies, and chromosomal abnormalities, particularly translocations, in either partner [1–4]. However, according to reports, in about a half of the cases seen at research centers, the cause remains unexplained despite conventional examinations conducted to identify the cause [5, 6]. Recently, we found that an abnormal embryonic karyotype was the most frequent cause of 2 or more RPL, accounting for as high as 41% of all the cases [7].

APS, acquired thrombophilia, is the only one treatable cause of RPL, and combined low-dose aspirin and heparin treatment having been shown to improve the live birth rate in patients with APS [8, 9]. Heritable thrombophilia has been reported to be associated with RM [10, 11].

Coagulation factor XII (FXII) is an 80-kDa serine protease that is involved in the initiation of the intrinsic pathway of the coagulation cascade. It is converted to its active form (activated factor XII, XIIa) by limited proteolysis [12], either by autoactivation on the surface of negatively charged compounds or by kallikrein [13]. Although FXII deficiency is associated with a prolonged activated partial thromboplastin time (aPTT), it is not associated with increased bleeding [14]. A C/T polymorphism has been identified in the promoter region of the *FXII* gene at nt46. The 46C/T polymorphism creates a new initiation codon (ATG) for transcription of the mRNA and a frameshift that produces a truncated protein. The T allele destroys the Kozak's consensus sequence (GCCAGCCATGG) for translation initiation signaling and prevents proper recognition of the translation initiation site. The T allele is therefore well-known to be associated with low plasma levels of factor XII [15]. The existence of associations between low FXII activity levels and thrombotic outcomes has been under debate for more than a decade.

We previously reported that the miscarriage rate of patients with low FXII activity (less than 39%) was significantly higher than that of patients with normal

FXII activity [16]. We also found that the frequency of the T allele did not differ between the women with a history of RPL and control fertile women [17]. However, the association between the C/T polymorphism or FXII activity and RPL could not be clearly elucidated, because the sample size was relatively small.

Thus, we conducted this cross-sectional and cohort study to determine the clinical significance of C/T polymorphism and FXII activity. We examined the association between 46C/T polymorphism and RPL, and between FXII activity and RPL in the cross-sectional study. We examined whether 46C/T polymorphism or FXII activity influenced the subsequent miscarriage rate in the cohort study. This was the first study to investigate the influence of *FXII* SNP on the subsequent pregnancy outcome.

Materials and Methods

Patients and controls

All patients were seen at Nagoya City University Hospital between September 2008 and July 2012. The study group consisted of 279 Japanese women with two or more consecutive pregnancy losses.

All patients underwent systematic examination, including hysterosalpingography, chromosome analysis of both partners, determination of aPL, including lupus anticoagulant (LA), by 5x-diluted aPTT, diluted Russel's viper venom time (RVVT) and β2 glycoprotein I-dependent anticardiolipin antibody determination (β2GPI-aCL), and blood tests for hypothyroidism and diabetes mellitus, before a subsequent pregnancy [18]. Criteria for exclusion from the analyses included the presence of uterine anomalies and chromosomal abnormalities in either partner. Patients with a history of thromboembolic events, pre-eclampsia, or abruptio placentae were also not included. The plasma samples for measurement of the FXII levels were obtained from the patients during the high phase of the basal body temperature (BBT).

Nine patients were positive for LA and 8 were positive for β2GPI-aCL. Of the 17, 7 patients were diagnosed as having APS, based on the persistence of the aPLs for more than 12 weeks.

Subsequent pregnancies of all patients were followed up until February 2013. Gestational age was calculated from BBT charts. Ultrasonography was performed once a week from 4 to 8 weeks of gestation. Dilation and curettage was performed in patients diagnosed as having miscarriage. A part of the villi was cultured, and the cells were harvested after 6–22 days of cultivation for chromosomal analysis using the standard G-banding technique.

Furthermore, 100 women with at least one child and no history of infertility or miscarriage were examined as controls. The control subjects consisted of 26 medical staff and 74 patients with dysplasia of the uterine cervix recruited from Nagoya City University Hospital from April to July 2013. None of the patients or controls were receiving any medication or were pregnant at the time of the study.

Ethics statement

This study was conducted with the approval of the Research Ethics Committee of Nagoya City University Graduate School of Medical Sciences. Each patient provided written consent after receiving a thorough explanation about the purpose of the study and the methods to be employed.

Cross-sectional study

The FXII activity was compared between the 17 patients with aPLs and 262 patients without aPLs in the cohort with RPL. The 17 patients with aPLs were excluded from further analysis. The characteristics of the 262 patients and 100 controls are shown in Table 1.

The allele frequencies of the CC, CT, TT genotypes of the *FXII* gene and FXII activity were compared between the 262 patients and 100 controls. We analyzed the data separately for the patients with a history of 3 or more early miscarriages before 10 weeks of gestation and those with a history of intrauterine fetal death (IUFD) in this cross-sectional study.

Cohort study

In the present cohort, the subsequent miscarriage rate was compared among the untreated 101 patients with a history of 3 or more early miscarriage with the CC, CT or TT genotype and according to the FXII activity.

A total of 39 of the 262 patients received heparin plus aspirin or aspirin alone in deference to the patient's wishes even after she has been provided information that aspirin or heparin had, in general, no effect on the live birth rate in cases of unexplained recurrent miscarriage [19]. These patients were excluded from the cohort study.

Statistical Analyses

The FXII activity between patients with and without aPLs was compared by student t-test.

The FXII activity was compared according to the genotype by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. The allele frequency was compared by the chi-squared test. Pearson's correlation coefficient was calculated between the aPTT and FXII activity, and between the age and FXII activity.

Multivariate logistic regression analyses were performed to examine the association of subsequent miscarriage, after adjusting for age and the number of previous miscarriages. FXII activity levels were categorized as high, normal or low using the 90^{th} percentile, 95^{th} percentile and 99^{th} percentile of the values in the controls. Furthermore, the FXII activity levels were also was categorized as high, normal and low according to the CC, CT and TT genotype using the 90^{th}

Table 1. Genotype and FXII activity in 17 patients with antiphospholipid antibodies.

	LA-aPTT	LA-RVVT	β2GPI dependent aCL	genotype	XII activity (%)
LA-aPTT-positive patients	9.8	negative	negative	CT	50
	8.7	negative	negative	CT	54
	8.3	negative	negative	CT	56
	8.2	negative	negative	CT	107
	10.9	negative	negative	TT	54
	9	negative	negative	TT	50
	8.1	negative	negative	TT	57
	8	1.3	negative	TT	53
	7.4	negative	negative	TT	65
Mean (SD) value					60.7 (17.9)
β2GPI-aCL-positive patients	negative	negative	4.6	CT	111
	negative	negative	2.8	CT	116
	negative	negative	2.4	CT	92
	negative	negative	2	CT	153
	negative	negative	10.7	TT	54
	negative	negative	7.6	TT	58
	negative	negative	5.4	TT	63
	negative	negative	2.3	TT	51
Mean (SD) value					87.3 (37.0)

10–90th percentile of FXII activity according to CC, CT and TT genotype were 101–141, 72–120 and 46–77.

doi:10.1371/journal.pone.0114452.t001

percentile of the values in the controls. The FXII activity levels were also categorized into quartiles of the patients.

For the post-hoc power analysis of the genotype frequency, we used a total of 362 subjects at an α of 0.05.

All the analyses were carried out using the statistical software SPSS, Version 21. $P<0.05$ was considered to denote statistical significance.

Genetic Analysis

Venous blood samples were collected in tubes containing K2 ethylenediamine tetraacetic acid and applied to genomic DNA extracting columns (QIAmp DNA blood Midi; Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed on genomic DNA samples using a Phusion High-Fidelity DNA Polymerase (NEW ENGLAND BioLabs, Finland). One μL (about 10 ng) solution (DNA preparation) was used as a template for the PCR. Exon 1 of the *FXII* gene was amplified by PCR using the sense and antisense primers 5′ CCAGTCCCACTATCTAGAAAAG-3′ and 5′ ATGGCTCATGGCTGT-GATAG-3′, respectively. After initial denaturation at 98℃ for 30 seconds, 35 cycles (98℃ for 10 seconds, 61.9℃ for 30 seconds, and 72℃ for 15 seconds) and final extension at 72℃ for 5 minutes were used to amplify 369-base pair products.

The substitution of 46C to T substitution is located 4 bases upstream from the translation initiation codon ATG, a region corresponding to the CseI (New Engrand BioLabs, Beverly, MA) restriction site (GACGC), which is therefore destroyed (46T). To analyze the polymorphism by electrophoresis, the samples were separated on 2% agarose gels (Takara, Japan) after enzyme digestion and stained with ethidium bromide.

To confirm the genotype, purified templates were sequenced with the BigDye Terminator v3.1 Cycle Sequencing kit (ABI Prism, Applied Biosystems, Foster City, CA, USA) on a 3100 automated sequencer.

Factor XII activity

Plasma samples were prepared in tubes containing 3.2% sodium citrate by centrifugation at 4℃ at 3,000 rpm for 10 minutes. The plasma samples were then stored at −40℃ until use. The FXII activity was determined by a clotting assay using coagulation factor XII kits (HemosIL, Instrumentation Laboratory, USA). A major domestic laboratory company performed the FXII activity measurements. The intra-assay CV for the high activity control was 2.98%, and that for the low activity control was 4.03%.

Results

Nine patients were positive for LA-aPTT and one was positive for both LA-aPTT and LA-RVVT (Table 1). The FXII activity in patients with LA was significantly lower than in the patients without LA (60.7±17.9% vs. 83.4±29.3%; p=0.02). FXII activity in 8 patients with β2GPI-aCL alone was 87.3±37.0 (not significantly different). Thus, we excluded the 17 patients with aPLs from the following comparison.

The mean age of the fertile controls was higher than that of the patients (p=0.002, Table 2). Eleven patients gave a history of previous intrauterine fetal death (IUFD). Secondary RPL was 18.7%.

The results of the cross-sectional study are shown in Table 3. The wild-type (CC), heterozygote (CT) and homozygote (TT) alleles for the *FXII* gene were observed in 22 (8.4%), 139 (53.1%) and 101 (38.5%) patients with RPL vs. 17 (17.0%), 38 (38.0%) and 45 (45.0%) controls. The frequency of CT in the patients with RPL was significantly higher than that in the controls (OR, 2.83; 95% CI, 1.37–5.85; p=0.005). The frequency of TT in the patients also tended to be higher than that in the controls. The statistical power for the frequency of CT was sufficient (1−β=0.79), while that for the frequency of TT was insufficient (1−β=0.31). The frequency of CT in the patients with 3 or more early miscarriage tended to be higher than that in the controls (OR, 2.32; 95% CI, 0.98–5.49; p=0.06).

In regard with the C/T ratio, the frequency of T allele in the patients was similar to that of controls.

Table 2. Characteristics of 262 patients with a history of recurrent pregnancy losses and 100 control healthy women with a history of live birth.

	Controls	2 or more	3 or more
No. of patients	100	262	121
Mean age (SD)	35.2 (4.7) 21–42	33.5 (4.6) 21–45	33.8 (4.8) 21–45
Mean (SD) No. of previous miscarriages	0	2.58 (0.79)	3.26 (0.69)
2		141	-
3		98	98
4		19	19
5		1	1
6		2	2
8		1	1
Mean (SD) No. of previous intrauterine fetal death	0	0.05 (0.23)	0.04 (0.24)
0		251 (95.8%)	117 (96.7%)
1–2		11 (4.2%)	4 (3.3%)
Mean (SD) No. of previous live births	1.6 (0.7)	0.20 (0.44)	0.26 (0.48)
Primary	0	213 (81.3%)	91 (75.2%)
Secondary	100	49 (18.7%)	30 (24.8%)

doi:10.1371/journal.pone.0114452.t002

The differences in the activity levels between the CC and CT ($p<0.001$), CT and TT ($p<0.001$) and CC and TT ($p<0.001$) genotypes in the patients, and between the CC and CT ($p<0.001$), CT and TT ($p<0.001$) CC and TT ($p<0.001$)

Table 3. Cross-sectional study: The frequencies of the CC, CT and TT genotypes and the factor XII activities in the 262 patients and 100 controls.

	The frequencies of the CC, CT and TT genotypes						
Genotype	Control n (%)	Patients with 2 or more miscarriage		Patients with 3 or more early miscarriage		Patients with a history of IUFD	
Total	100	262	OR (95% CI)	117	OR (95% CI)	11	OR (95% CI)
CC	17 (17)	22 (8.4)	reference	11 (9.4)	reference	0	reference
CT	38 (38)	139 (53.1)	**2.83 (1.37–5.85) 0.005**	57 (48.7)	2.32 (0.98–5.49) 0.056	8 (72.7)	-
TT	45 (45)	101 (38.5)	1.73 (0.84–3.58) 0.136	49 (41.9)	1.68 (0.71–3.98) 0.235	3 (27.3)	-
CT, TT	83 (83)	240 (91.6)	**2.23 (1.13–4.41) 0.021**	106 (90.6)	1.97 (0.88–4.44) 0.100	11 (100)	-
C/T ratio	0.36/0.64	0.34/0.66		0.34/0.66		0.36/0.64	
	The factor XII activities: mean (SD) value and range						
Total XII activity	83.8 (28.6) 40–143	83.8 (29.1) 37–178		80.9 (29.0) 37–145		71.8 (14.4) 51–94	
CC	123.1 (14.3) 97–143	126.1 (17.7) 87–160		125.2 (14.1) 97–143		-	
CT	94.3 (18.5) 55–129	89.2 (25.8) 37–178		89.9 (22.4) 37–145		74.5 (12.5) 61–94	
TT	60.0 (14.5) 40–115	65.8 (21.6) 37–139		61.6 (17.8) 37–137		64.7 (19.5) 51–87	

doi:10.1371/journal.pone.0114452.t003

genotypes in the controls were significant. ANOVA revealed no significant difference in the mean FXII activity levels between the patients and controls. Twenty-seven of the 262 (10.3%) patients and 10 of the 100 (10%) controls showed decreased FXII activity (<50%), the difference in the percentage not being significant. A weak correlation coefficient between the aPTT and FXII activity was observed (r=−0.479). There was no correlation between the FXII activity and age.

The subsequent miscarriage rate in patients with RPL was 26.0% (68/262). A total of 32 (47.1%) miscarried conceptuses could be karyotyped, of which, 15 (46.9%) had a normal karyotype and 17 (53.1%) had an abnormal karyotype.

Association between the subsequent miscarriage rate and the *FXII* genotype or FXII activity in the 101 untreated patients with a history of RM was analyzed (Table 4). The miscarriage rates were 30.0%, 21.2% and 30.8% in the patients with the CC, CT and TT genotypes, respectively. According to the results of the logistic regression analysis, there was no increase in the miscarriage rate associated with the presence of CT and TT as compared to that associated with the presence of CC. A similar result was obtained after excluding cases with miscarriage caused by an abnormal embryonic karyotype.

The logistic regression analysis also showed no significant increase in the miscarriage rate associated with low or high FXII activity levels, classified using the cutoff values of 10–90th percentile, 5–95th percentile and 1–99th percentile of the control values (Table 4). The frequencies obtained using the latter two cutoffs seemed to be insufficient to detect any significant differences (not shown). The multivariable analyses showed no significant increase in the miscarriage rate associated with FXII activity levels even after excluding cases with an abnormal embryonic karyotype.

Furthermore, when the FXII activity was categorized into high, normal or low classified using the 10–90th percentile of the control values for each of the CC, CT and TT genotypes, the logistic regression analysis showed significant increase in the miscarriage rate associated with high FXII activity level (OR, 5.65; 95% CI, 1.24–25.64; p=0.03). However, the difference disappeared after exclusion of cases with an abnormal embryonic karyotype. When the FXII activity was categorized into quartiles, the intermediate level, that is, a FXII activity level of 85–101%, predicted subsequent miscarriage (OR, 4.65; 95% CI, 1.06–20.4; p=0.04). The result remained significant after excluding cases with an abnormal embryonic karyotype.

Discussion

The FXII activity in the 9 patients with LA was significantly lower than that in the patients without LA in the present study. This was in line with the results of Gallimore's study [20]. LA might include antibodies to factor XII, as an association has been reported between the presence of antibodies to factor XII and recurrent fetal loss in patients with APS [21]. In the present study, there was no

Table 4. Cohort study: Subsequent miscarriage rate according to the genotype and FXII activity in 101 untreated patients with a history of recurrent miscarriage.

		Miscarriage rate	Crude analysis		Multivariable Logistic regression		Miscarriage rate excluding abnormal embryonic karyotype	Crude analysis		Multivariable Logistic regression	
			OR [a] (95% CI [b])	P-value	OR (95% CI)	P-value		OR (95% CI)	P-value	OR (95%CI)	P-value
Genotype	CC	30.0% (3/10)	reference		reference		22.2% (2/9)	reference		reference	
	CT	21.2% (11/52)	0.40 (0.08–1.96)	0.26	0.40 (0.07–1.96)	0.26	19.6% (10/51)	0.86 (0.15–4.76)	0.86	0.50 (0.08–3.06)	0.46
	TT	30.8% (12/39)	1.04 (0.23–4.72)	0.96	0.79 (0.17–3.73)	0.77	20.6% (7/34)	0.90 (0.15–5.38)	0.92	0.68 (0.11–4.18)	0.68
FXII activity (10–90th percentile)[c]	Normal	31.3% (22/83)	reference		reference		20.8% (16/77)	reference		reference	
	High	37.5% (3/8)	1.66 (0.37–7.52)	0.51	1.96 (0.41–9.35)	0.40	28.6% (2/7)	1.52 (0.27–8.62)	0.63	1.84 (0.31–10.99)	0.50
	Low	10.0% (1/10)	0.29 (0.04–2.57)	0.28	0.35 (0.04–3.01)	0.34	10.0% (1/10)	0.42 (0.05–3.60)	0.43	0.50 (0.06–4.31)	0.52
Genotype and FXII activity (10–90th percentile)[d]	Normal	25.3% (19/75)	reference		reference		21.1% (15/71)	reference		reference	
	High	66.7% (6/9)	**5.88 (1.34–25.64)**	**0.02**	**5.65 (1.24–25.64)**	**0.03**	50.0% (3/6)	7.75 (0.68–20.41)	0.28	4.22 (0.73–24.4)	0.11
	Low	5.9% (1/17)	0.18 (0.02–1.48)	0.11	0.20 (0.02–1.62)	0.13	5.9% (1/17)	0.23 (0.03–1.90)	0.17	0.24 (0.03–2.07)	0.24
FXII activity (quartile)	−56	10.3% (3/29)	reference		reference		7.1% (2/28)	reference		reference	
	57–84	31.8% (7/22)	4.05 (0.91–18.18)	0.07	3.60 (0.78–16.40)	0.10	25.0% (5/20)	4.33 (0.75–25.00)	0.10	3.86 (0.64–23.26)	0.14
	85–101	36.0% (9/25)	**4.88 (1.15–20.83)**	**0.03**	**4.67 (1.06–20.41)**	**0.04**	33.3% (8/24)	**6.49 (1.22–34.48)**	**0.03**	**6.37 (1.15–34.48)**	**0.03**
	102–	28.0% (7/25)	3.37 (0.77–14.71)	0.11	3.23 (0.71–14.49)	0.13	18.2% (4/22)	2.89 (0.48–17.54)	0.25	2.76 (0.44–17.54)	0.28

a; odds ratio, b; confidence interval, c; Normal 50–127, High 128-, Low -49,
d; CC 101–141, CT 72–120, TT 46–77.

doi:10.1371/journal.pone.0114452.t004

difference in the FXII activity levels between the patients and controls after patients with LA were excluded.

In our previous study, we failed to show an association between LA and reduced FXII activity levels because we compared only patients with the TT genotype [17]. We previously reported that low FXII activity, but not an associated common genetic polymorphism, 46C/T, was linked to RPL [17]. A systematic review concluded that FXII deficiency is associated with RM [22]. Pauer et al. reported that 10 of 67 women with primary recurrent abortion (14.9%) and 4 of 33 women (12.1%) with secondary recurrent abortion had reduced factor XII activity (<60%), while all controls had normal FXII activity [23]. However, they checked for only anticardiolipin antibody, and not LA. These results could be attributable to the inclusion of patients with LA.

Low levels of FXII was confirmed not to influence the subsequent miscarriage rate when the cutoff values were based on the 90^{th} percentile, 95^{th} percentile and 99^{th} percentile of the control values. We previously reported that the miscarriage rate in patients with low FXII activity levels (less than 39%) was significantly higher than that in the patients with normal activity levels [16]. The sample size in our previous study was relatively small, because 4 of the 5 untreated patients with FXII activity levels of less than 39% developed miscarriage. The present study included the largest sample size, and was the first prospective cohort study.

CT genotype, but not the TT genotype was confirmed to be a risk factor for RM. We found no association between 46C/T polymorphism and RPL in a previous study, because the frequency of this SNP was similar between the patients and controls (0.31/0/69 and 0.31/0.69) [17]. We did not conduct comparisons according to the genotype distribution, although the frequencies of CC, CT and TT in the patients and controls were 9.6%, 43.4% and 47.0% and 16.4%, 29.9% and 53.7%, respectively, in our previous study, being quite similar to those in the present study.

Walch et al. demonstrated that the genotype distribution was not significantly different between a RM group of 212 patients and a control group of 149 women in Middle-European Caucasian population [24]. It is speculated that the distribution of the risk alleles might show ethnic differences.

Johnson et al. found a very weak association between myocardial infarction and the CT+TT genotype (OR 1.13, 95% CI 1.00–1.27), and suggested that this was caused by low FXII level activity because of the T allele [25]. We found a similar association between the CT+TT genotype and RPL (Table 3), however, only CT was significant, not TT. The statistical power for the frequency of CT was sufficient ($1-\beta=0.79$). The statistical power for the frequency of TT was insufficient and ($1-\beta=0.31$). This implies that a larger study might have possibilities to show the significant of differences for the TT genotype.

That the T allele might act via other mechanism(s), such as endothelial dysfunction, and not via low FXII activity, to induce myocardial infarction and miscarriage. LaRusch found that FXII initiates signaling to induce human umbilical vein endothelial cell proliferation, growth and angiogenesis [26]. FXII as a growth factor stimulates angiogenesis after ischemia, inflammation, and injury,

just like vascular endothelial growth factor. FXII plays various roles in vivo: FXII triggers the plasma contact system via the kallikrein kinin-system and intrinsic coagulation pathway, and zymogen FXII functions as a growth factor that mediates cell signaling leading to proliferation and stimulation of angiogenesis. FXII might have another role of maintaining pregnancy.

Low levels of FXII did not increase the subsequent miscarriage rate when the cutoff values were based on the 90^{th} percentile, 95^{th} percentile and 99^{th} percentile of the control values. Activity levels of 85–101%, which fall in the second highest quartile, and not low FXII activity levels, were found to predict subsequent miscarriage. This intermediate range might correspond to the CT genotype, because the reported mean (SD) FXII activity in patients with the CT genotype is 89.2 (25.8), even though the CT genotype itself did not influence the likelihood of subsequent miscarriage.

Many cross-sectional studies have been reported on associations between polymorphism and RPL, such as 4G/4G for plasminogen activator inhibitor-1 polymorphisms (OR 11.0, 95% CI 2.3–52.4), protein Z intron F G79A polymorphism (OR 0.3, 95% CI 0.1–0.8) and Annexin A5's -1C/T (OR 2.7, 95% CI 1.0–6.7) [27–29]. However, the clinical significance could not be established because most were not cohort studies. Our cross-sectional study confirmed that variations in the *ANXA5* gene upstream region, especially SNP5, were risk factors for RPL, and our cohort study concluded that the presence/absence of the *ANXA5* risk allele did not have any significant predictive effect on the subsequent pregnancy outcome [30].

FXII activity level in the intermediate range, that is, 85–101% was predictive of subsequent miscarriage. The FXII activity is increased in old age, in females, during pregnancy, during intake of an oral contraceptive. The influence of age could be ignored, although all the patients were younger than the control women, because there was no correlation between the FXII activity and age in the present study. We could not measure FXII activity in duplicate. These are some limitations of the present study. Further study is needed because this was a new finding.

The CT genotype of the *FXII* gene was confirmed to be a risk factor for RPL, but it was not shown to serve as a reliable clinical predictor of the subsequent pregnancy outcome. Therefore, we propose that testing for this allele is not needed, as it is without clinical benefit and is an unnecessary expense.

Author Contributions

Conceived and designed the experiments: MS-O. Performed the experiments: EA CY-N MN NS. Analyzed the data: EA TE MS-O. Contributed reagents/materials/analysis tools: TK KK YO MS-O. Wrote the paper: EA MS-O.

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Molecular Cell
Article

Necessary and Sufficient Role for a Mitosis Skip in Senescence Induction

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http://dx.doi.org/10.1016/j.molcel.2014.05.003

SUMMARY

Senescence is a state of permanent growth arrest and is a pivotal part of the antitumorigenic barrier in vivo. Although the tumor suppressor activities of p53 and pRb family proteins are essential for the induction of senescence, molecular mechanisms by which these proteins induce senescence are still not clear. Using time-lapse live-cell imaging, we demonstrate here that normal human diploid fibroblasts (HDFs) exposed to various senescence-inducing stimuli undergo a mitosis skip before entry into permanent cell-cycle arrest. This mitosis skip is mediated by both p53-dependent premature activation of APC/C^{Cdh1} and pRb family protein-dependent transcriptional suppression of mitotic regulators. Importantly, mitotic skipping is necessary and sufficient for senescence induction. p16 is only required for maintenance of senescence. Analysis of human nevi also suggested the role of mitosis skip in in vivo senescence. Our findings provide decisive evidence for the molecular basis underlying the induction and maintenance of cellular senescence.

INTRODUCTION

The inability of cultured human cells to proliferate indefinitely, ending in cellular senescence, was first described by Hayflick and Moorhead (1961). Subsequently, several lines of evidence revealed that cellular senescence was also triggered by diverse genotoxic stimuli, including telomere dysfunction, activated oncogenes, reactive oxygen species (ROS), and DNA damage (Kuilman et al., 2010). Senescence is now believed to play a critical role in suppression of tumorigenesis as well as in aging-related changes in various organs resulting from a permanent loss of proliferation capacity (Campisi and d'Adda di Fagagna, 2007; Halazonetis et al., 2008).

Cellular senescence requires functional p53 and pRB family proteins, both of which regulate growth signaling. This may explain why these genes are often mutated in a vast majority of human cancers (Burkhart and Sage, 2008; Levine and Oren, 2009). This is supported by the fact that viral oncoproteins that can inhibit either p53 or pRB family proteins allow cells to bypass cellular senescence (Shay et al., 1991). Although the precise roles of these tumor suppressors in the senescence process are incompletely understood, various models of senescence induction have been proposed (Adams, 2009; Courtois-Cox et al., 2008; Rufini et al., 2013). One such proposal is that senescence-inducing stimuli ultimately trigger a DNA damage response (DDR) that in turn activates p53. p21 (*CDKN1A*), a p53-target gene, is expressed and arrests cells at the G1 phase of the cell cycle by preventing phosphorylation and inactivation of pRB through inhibition of G1 and S phase Cdk activities (Cobrinik, 2005). pRb phosphorylation is also suppressed by another Cdk inhibitor, p16 (*CDKN2A*), that is upregulated during the senescence process (Rayess et al., 2012). Hypophosphorylated pRb suppresses transcription of canonical E2F (E2F1– E2F3) target genes to arrest the cell cycle at G1 (Rowland and Bernards, 2006).

In contrast, the accumulation of G2 phase cells during replicative senescence has also been reported, arguing against the senescence model described above (Mao et al., 2012; Ye et al., 2013). In addition, p21-mediated inhibition of Cdk1 and Cdk2 was proposed to prematurely activate APC/C^{Cdh1} to destroy various APC/C substrates, resulting in long-term growth arrest at G2 in response to genotoxic stress (Baus et al., 2003; Wiebusch and Hagemeier, 2010). Thus, the fundamental basis for senescence induction and the phases at which senescent cells exit the cell cycle remain controversial. The factors that determine whether cells will undergo senescence (terminal growth arrest) versus transient cell-cycle arrest, also remain largely elusive.

In this study, we have analyzed the senescence process induced by various stimuli using time-lapse live-cell imaging. We found that the majority of cells underwent a mitosis skip before permanently exiting the cell cycle. This mitotic skipping appears to be necessary and sufficient for the induction of senescence both in vitro and in vivo.

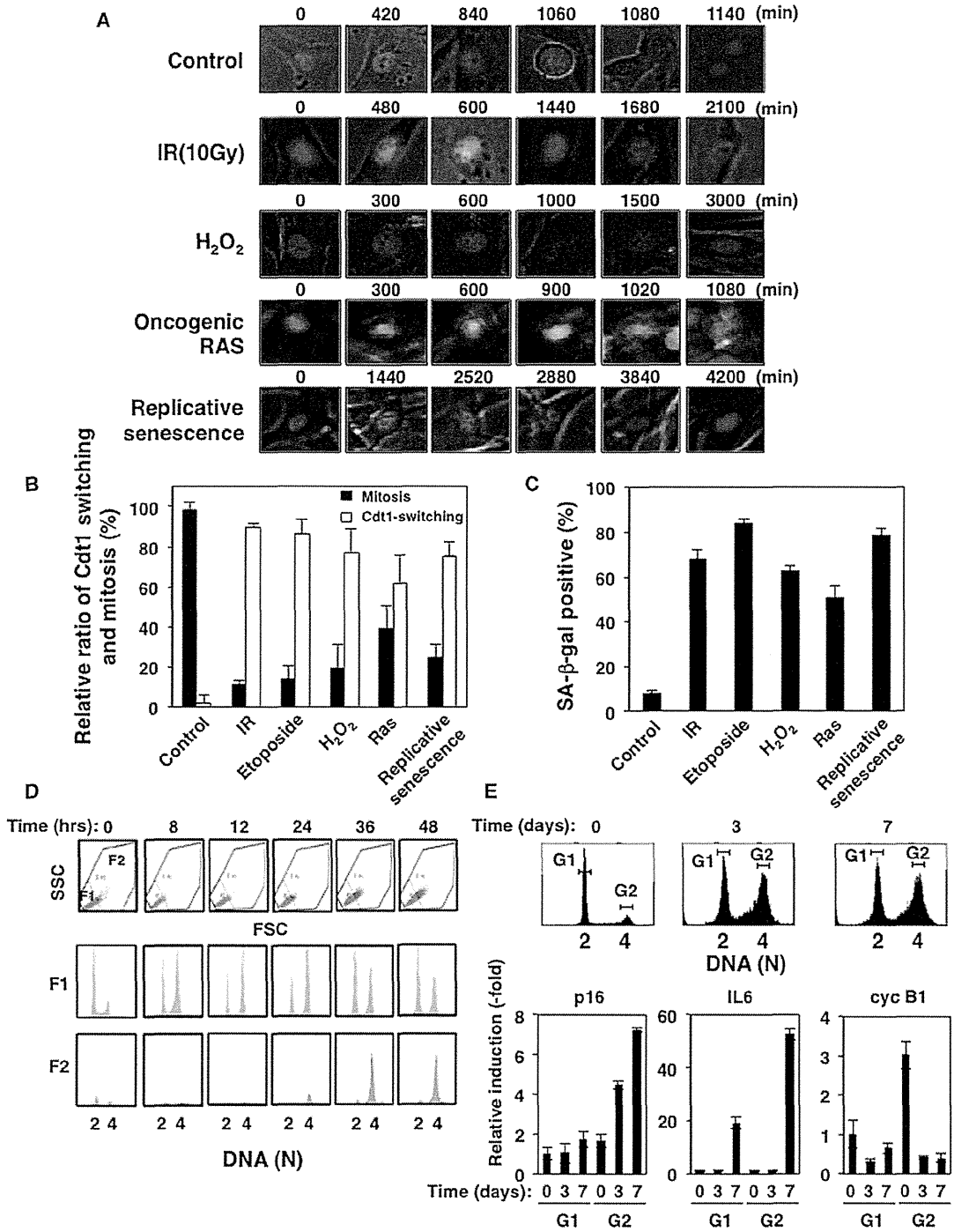

Figure 1. Senescent Cells Are Mononucleated Tetraploid G1 Cells Triggered by a Mitosis Skip

(A) Young (PD 8) and near senescent (PD > 60) HCA2 cells (FUCCI-HCA2 cells) were infected with FUCCI lentiviruses expressing mKO2-hCdt1 (red) and mAG-hGeminin (green). Young cells were untreated (Control) or treated with IR (10 Gy), H_2O_2 (50 μM) for 48 hr or etoposide (200 nM) for 48 hr. Expression of oncogenic RAS was induced by the addition of doxycycline to young cells expressing Tet-on 3×Flag-H-Ras^{val12}. Replicative senescence was analyzed using near senescent cells. The resulting cells were imaged 3 days after treatment. Representative images at the indicated times are shown.

(B) The relative ratio of Cdt1-switching cells versus total cells changings from green to red color was determined by counting at least 100 cells treated as in (A). Data are presented as means ±SD of at least three independent experiments.

(C) SA-β-gal-positive cells were identified using cells 6 days after treatment as in (A). Data are presented as means ±SD of at least three independent experiments.

(legend continued on next page)