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STUDY PROTOCOL Open Access

Metformin efficacy and safety for colorectal polyps: a double-blind randomized controlled trial

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Abstract

Background: Colorectal cancer is one of the major neoplasms and a leading cause of cancer death worldwide, and new preventive strategies are needed to lower the burden of this disease. Metformin, a biguanide, which is widely used for treating diabetes mellitus, has recently been suggestive to have a suppressive effect on tumorigenesis and cancer cell growth. In a previous study conducted in non-diabetic subjects, we showed that oral short-term low-dose metformin suppressed the development of colorectal aberrant crypt foci (ACF). ACF have been considered as a useful surrogate biomarker of CRC, although the biological significance of these lesions remains controversial. We devised a prospective randomized controlled trial to evaluate the chemopreventive effect of metformin against metachronous colorectal polyps and the safety of this drug in non-diabetic post-polypectomy patients.

Methods/Design: This study is a multi-center, double-blind, placebo-controlled, randomized controlled trial to be conducted in non-diabetic patients with a recent history of undergoing colorectal polypectomy. All adult patients visiting the Yokohama City University hospital or affiliated hospitals for polypectomy shall be recruited for the study. Eligible patients will then be allocated randomly into either one of two groups: the metformin group and the placebo group. Patients in the metformin group shall receive oral metformin at 250 mg per day, and those in the placebo group shall receive an oral placebo tablet. At the end of 1 year of administration of metformin/placebo, colonoscopy will be performed to evaluate the polyp formation.

Discussion: This is the first study proposed to explore the effect of metformin against colorectal polyp formation. Metformin activates AMPK, which inhibits the mammalian target of rapamycin (mTOR) pathway. The mTOR pathway plays an important role in the cellular protein translational machinery and cell proliferation. Patients with type 2 diabetes taking under treatment with metformin have been reported to be at a lower risk of cancer development than those not taking under treatment with metformin. We showed in a previous study that metformin suppressed the formation of human colorectal ACF. We therefore decided to conduct a study to determine whether metformin might suppress the formation of human colorectal polyps.

Trial registration: This trial has been registered in the University hospital Medical Information Network (UMIN) Clinical Trials Registry as UMIN000006254

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Background

Colorectal cancer (CRC) is a major neoplasm worldwide [1], and both its prevalence and mortality have been increasing [2]. Removal of colorectal polyps has been to shown to reduce the risk of future development of colorectal cancer and advanced adenoma [3,4]. On the other hand, patients with polyps (adenomas and/or hyperplastic polyps) constitute a high-risk group for the development of metachronous colorectal polyps and/or CRC [5]. Therefore, a paradigm shift from surveillance for early detection of cancer or adenomas (polypectomy) to new strategies for prevention, including chemoprevention, is needed to lower the burden of this disease. Several large epidemiologic and/or clinical studies have evaluated the possible preventive effects of more than 200 agents, including fiber, calcium, and non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin and selective cyclooxygenase-2 (COX-2) inhibitors, in protecting against CRC development [6]. NSAIDs, especially COX-2 inhibitors, administered either alone or in combination with other agents, have shown the most promise, until date, for CRC risk reduction [4], although reports have revealed an increased risk of serious cardiovascular events associated with the use of COX-2 inhibitors [7,8]. In light of the adverse cardiovascular effects of COX-2 inhibitors and lack of demonstrable efficacy of the other agents that had initially shown promise in this setting, novel drugs that would be both safe and effective for CRC prevention need to be developed. CRC is associated with lifestyle-related diseases, such as diabetes mellitus and obesity [9-12], therefore, we considered that these conditions might represent potential new targets for CRC chemoprevention.

Metformin (1,1-dimethylbiguanide hydrochloride) is a biguanide derivative that has long been used widely for treating diabetes mellitus [13]. It decreases basal glucose output by suppressing gluconeogenesis and glycogenolysis in the liver and increasing glucose uptake by the muscle. Because metformin does not directly stimulate insulin secretion, it is associated with a lower risk of hypoglycemia than other oral antidiabetic drugs [14]. The molecular mechanism involved in the action of metformin is liver kinase B-1dependent activation of AMP-activated protein kinase (AMPK) [15]. Patients with type 2 diabetes under treatment with metformin have been reported to be at a lower risk of cancer development (including CRC) than those not under treatment with metformin [16,17]. This evidence suggests that metformin might be a candidate agent for CRC chemoprevention in diabetic patients. However, since diabetes mellitus itself is a risk factor for cancer, treatment of diabetes mellitus may reduce the risk; therefore, it is still unclear whether the suppressive effect of metformin against CRC may be exerted by the direct chemopreventive effect of the drug or be mediated by its antidiabetic effect. Therefore, we considered that in order to validate the chemopreventive effect of metformin, a clinical trial in nondiabetic patients needs to be conducted.

In previous studies, we demonstrated the chemopreventive effect of metformin in two rodent models (a genetic model and a chemically-induced cancer model) and one human study of colorectal carcinogenesis. We showed that metformin suppressed the development of intestinal polyps in adenomatous polyposis coli (APC$^{Min/+}$) mise, a murine model of familial adenomatous polyposis [18]; furthermore, we showed that metformin suppressed azoxymethane-induced formation of colorectal aberrant crypt foci (ACF) by activating AMPK [19]. Both studies were performed in nondiabetic mice, which suggested the direct chemopreventive potential of metformin per se. In the study conducted on nondiabetic human subjects, we showed that oral low-dose administration of metformin (250 mg per day) suppressed the formation of colorectal ACF and that the drug was safe [20]. ACF are considered as a reliable surrogate biomarker of CRC [21], although their biological significance still remains controversial. Therefore, in CRC chemoprevention trials, in general, the incidence of polyps or of the cancer itself is set as the study endpoint. Although the incidence rate of CRC would be the most reliable endpoint, use of this endpoint would be unsuitable for chemoprevention trials, because of the relatively low occurrence rate of CRC in the general population [22] and the long-term observation period that it would necessitate. Moreover, observation of polyps detected in annual colonoscopies until they grow into cancer would be fraught with ethical problems. Thus, we set the appearance of colorectal polyps as a suitable endpoint for our chemopreventive trial.

Thus, we devised a prospective randomized controlled trial to evaluate the chemopreventive effect of metformin against the development of metachronous colorectal polyps and the safety of this drug in nondiabetic post-polypectomy patients.

This is the first clinical trial of metformin as a chemopreventive agent against metachronous colorectal polyps in humans.

Methods/Design

Study design and setting

This study is designed as a multi-center, double-blind, placebo-control, randomized controlled trial to be performed in nondiabetic patients with a recent history of undergoing colorectal polypectomy. The study will take place at the Division of Gastroenterology, Yokohama City University Hospital, and its 5 affiliate hospitals. The coordinating office shall be at Yokohama City University

Hospital, with the registration, randomized allocation and data collection to be conducted at this site.

Ethical considerations and registration

The study protocol is in compliance with the Declaration of Helsinki [23] and the Ethics Guidelines for Clinical Research published by the Ministry of Health, Labour, and Welfare, Japan [24]. We obtained approval for this study from the Ethics committee of Yokohama City University Hospital on July 8th 2011. The protocol and informed consent forms were approved by the institutional ethics committee at each of the participating institutions. This trial has been registered in the University hospital Medical Information Network (UMIN) Clinical Trials Registry as UMIN000006254. Written informed consent for participation in the study will be obtained from all the participating patients. The trial results will be reported in conformity with the Consolidated Standards of Reporting Trials (CONSORT) 2010 guidelines [25].

Eligibility criteria

All adult patients visiting the hospital for polypectomy will be recruited for the study.

The inclusion criteria are as follows:

1) No colorectal polyps present after the polypectomy

2) Age 40 to 80 years as on the date of informed consent

3) Willingness to provide written informed consent

The likelihood of development of colorectal polyps in the young is low and the diagnosis history of polyps in the young is usually related to familial adenomatous polyposis or hereditary non-polyposis colorectal cancer; on the other hand, the elderly have various complications. This was the rationale for our setting the age criterion for inclusion in this study as 40 to 80 years.

The exclusion criteria are as follows:

1) History of diabetes mellitus (use of medication and/or HbA1c over 6.5%)

2) History of regular use (defined as at least once per week) of NSAIDs and/or aspirin

3) History of bowel surgery

4) History of malignant disease (excluding carcinoma in adenoma, carcinoma in situ that has already been resected)

5) History of heart failure, renal failure, liver cirrhosis or chronic hepatic failure

6) History of familial adenomatous polyposis

7) History of hereditary non-polyposis colorectal cancer

8) History of inflammatory bowel disease

9) Pregnancy or possibility of pregnancy

10) Patients judged as inappropriate candidates for the trial by the investigators

Intervention

All eligible patients will be allocated randomly to one of two groups, the metformin group and the placebo group. Endoscopists, doctors at the follow-up outpatient clinics and patients will be blinded to the allocation. Patients in the metformin group shall receive oral metformin at 250 mg per day, and those in the placebo group shall receive oral placebo tablet. At the end of 1 year of administration of metformin/placebo, colonoscopy will be performed to evaluate the polyp formation.

Outcome measurements

The primary endpoint shall be the prevalence of colorectal polyps and number of polyps after 1-year's intervention. The endoscopic examinations and polypectomies will be performed using Olympus colonoscopes (model H260AZI). The day before the endoscopy, each patient will be instructed to consume a low-residue diet and shall receive 5 mg of oral sodium picosulfate. On the day of the endoscopy, the patients shall receive 2000 ml of polyethylene glycol (PEG). If the feces are not sufficiently clear, an additional 1000 - 2000 ml of PEG may be given to ensure sufficient bowel cleaning. At the time of the polypectomies, the endoscope shall be inserted into the cecum, and the entire colorectum will be carefully observed as the endoscope is pulled back. If any polyps are detected, polypectomy will be performed. At the end of 1 year of administration of metformin/placebo, the same endoscopists will perform the repeat endoscopic examinations. If a polyp(s) is detected at the repeat colonoscopy (after treatment for one year), a biopsy will be performed. A total of 6 endoscopists from Yokohama City University Hospital and the 5 affiliate hospitals will perform the polypectomies and endoscopic examinations. All procedures will be recorded on DVD, and all the polyps will be photographed. The number of polyps in each patient will first be counted by the operators during the performance of the colonoscopy. To further ensure validity, the number of polyps will be counted again through observation of the recorded DVD by 3 blinded expert endoscopists (H.T, H.E, and E.S). If these expert endoscopists judge the colonoscopic examination as having been inadequate in any case, that case will be excluded. The biopsied polyps will be evaluated by expert pathologists (Y.N and S.Y).

The secondary outcomes are (1) the drug safety; adverse events will be monitored by the doctor at every follow-up visit to the outpatient clinic. Adverse events will be graded according to the National Cancer Institute Common Toxicity Criteria for Adverse Events (NCI-CTCAE), version 4.0. Diarrhea, the most frequent adverse event related to use of metformin, will be watched for spontaneous resolution within a few days and/or managed by antiflatulent drugs. If Grade 3 or more severe adverse events appear,

the follow-up doctor shall report it (them) to the coordinating office and the case will be withdrawn from the study at that point; (2) laboratory data (fasting blood glucose, fasting blood insulin, HbA1c, total cholesterol, LDL-cholesterol, blood urea nitrogen (BUN), creatinine); (3) the number of ACF. At the time of the 1-year colonoscopy and polypectomy, the lower rectal region from the middle Houston valve to the dentate line will be washed thoroughly with water, sprayed with 0.25% methylene blue, which would be left to stand for 2 min, then again washed thoroughly with water. The number of rectal ACF will be counted with a magnifying endoscope [21]; (4) physical examination findings (body weight, body mass index (BMI)). Metformin is widely used as an antidiabetic drug that improves insulin resistance. The effect of metformin on insulin resistance and the plasma lipid profile will be evaluated by comparing these parameters measured at the baseline and at 1 year in the metformin group and placebo group. All participants will receive physical examination and laboratory tests at the time of the 1-year endoscopic examination and polypectomy; (5) effects of metformin on the cell-proliferative and apoptotic activities in the rectal epithelium and polyps (if any). Colonic epithelial samples will be obtained from the same trial patients by biopsy at the time of the 1-year colonoscopy and polypectomy. The cell-proliferative activity will be evaluated by staining for the proliferative cell nuclear antigen (PCNA), and the cell-apoptotic activity by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method; (6) expression levels of protein (AMPK, mTOR/S6K) in the rectal epithelium and polyps (if any) that are thought to be pharmacological targets of metformin. The expression levels of these proteins shall be determined by western blot analysis.

Randomization

The investigator shall convey the patient's details to the central registration centre via fax. After an eligibility check, the patients will be randomly assigned to receive metformin or placebo at the central registration centre by a computer program, using a minimization method, with stratification by institute, age, gender and BMI. In this way, the patient assignment will be concealed from the investigator. The randomization center will allocate a numbered treatment pack to each patient, which will contain all the drugs or placebos needed to complete a course of the trial treatment for one patient.

Drug supply

Metformin will be purchased from Dainippon Sumitomo Parma Co., Ltd. The placebo (250 mg lactose) will be purchased from Kokando Co., Ltd, Toyama, Japan. All trial drugs will be packaged identically and identified only by number. Subjects will be instructed to take one tablet of the trial drug after breakfast every day, and to visit the hospital every 4 weeks for evaluation of the subjective symptoms and for receiving a new supply of medication. Compliance will be monitored by counting the empty drug packages returned by the patients at every visit to the outpatient clinic.

Sample size estimation

We previously showed that metformin administered at 250 mg/d for 1 month directly suppressed the formation of ACF. In that study, mean number of ACF per patient decreased significantly from 8.78 ± 6.45 (baseline) to 5.11 ± 4.99 (at 1 month, $p = 0.007$) [20]. Based on a similar study, Takayama et al. reported that sulindac administration at 300 mg/d for 2 months to post-polypectomy patients suppressed ACF formation, decreasing the number of ACF from 7.70 ± 4.04 (baseline) to 4.00 ± 2.95 (at 2 months, $p < 0.001$) [26]. From these reports, we estimated that metformin and sulindac may have equivalent effect on suppression of ACF formation. Moreover, from the same study, Takayama et al. reported that in the post-polypectmoy patients who received 2-months' intervention, the number of polyps (hyperplastic polyp and adenoma) at 1 year after the treatment was significantly lower in the sulindac group in comparison with that in the placebo group (26/48 (54.2%) vs. 15/48 (31.3%), $p = 0.025$) [26]. Therefore, we speculated that 1-year's treatment with metformin might yield equivalent suppression of metachronous polyp formation to that with sulindac. To detect the reduction in the number of metachronous polyps in the metformin group using the chi-square test with a two-sided significance level of 5% and a power of 80%, it was found that a sample size of 68 patients per group would be necessary. Assuming a 10% dropout rate, we propose to recruit 75 patients per group, that is, a total of 150 patients.

Statistical analysis

The prevalence of polyps in each group, the primary endpoint, will be compared between the metformin group and placebo group by the chi-square test. The safety, one of the secondary endpoints, will be similarly compared by the chi-square test. The remaining results in the two groups will be compared by the Mann-Whitney U test or Student's t test. A P value of < 0.05 shall be regarded as indicative of significance. The analysis will be performed using SPSS, version 17.0 (SPSS Inc., Chicago, Il.).

Trial steering committee and data monitoring committee

The Trial Steering Committee and Data Monitoring Committee shall be located at the Department of Clinical Research, Yokohama City University School of Medicine. The committee shall consist of three people: Yutaka

Natsumeda, M.D., Satoshi Inoue, M.D., and Yukiharu Yamaguchi, Ph.D. The Management Team will monitor the trial progress status and data by face-to-face and/or telephonic contact with each of the six sites every month.

Study flow

A flow chart of the study is shown in Figure 1.

Discussion

This is the first study proposed to explore the effect of metformin against colorectal polyp formation. Metformin activates AMPK, which inhibits the mammalian target of rapamycin (mTOR) pathway [15]. The mTOR pathway plays an important role in the cellular protein translational machinery and cell proliferation [27]. The best-characterized downstream effector of mTOR is S6 kinase, which regulates the initiation and elongation phases of translation [28]. Activation of the mTOR pathway has been shown to accelerate cell cycle progression from G1 to S in CRC DLD-1 cells [29]. Therefore, AMPK activation may inhibit cell growth and proliferation by suppressing protein synthesis, thereby having a potent antiproliferative effect. Recent evidence indicates that metformin has a suppressive effect on tumorigenesis and cancer cell growth [30-32]. In one study, metformin was demonstrated to activate AMPK and consequently decrease cellular proliferative activity, to produce a general decrease in protein synthesis in vitro in human breast carcinoma cells [30]. Metformin was also shown to inhibit the proliferation of human prostate cancer cells [32].

This trial may have the following limitations. First, we do not propose to conduct a dose-response study of the effect of metformin on colorectal polyp formation. Until now, trails of metformin for cancer prevention and adjuvant treatment have been conducted using high-dose metformin (500 - 2000 mg per day). However, high-dose metformin use is associated with the risk of development of lactic acidosis and gastrointestinal adverse effects (including diarrhea). Gontier et al. reported from a PET/CT study, that subjects treated with antidiabetic agents, including metformin, showed high and diffuse bowel uptake of 18 F-FDG [33]. This suggests that AMPK is present in abundance in the bowel epithelium and that activation of AMPK by metformin up-regulates the expression of glucose transporters. We showed in a previous study that oral low-dose metformin (250 mg per day) suppressed

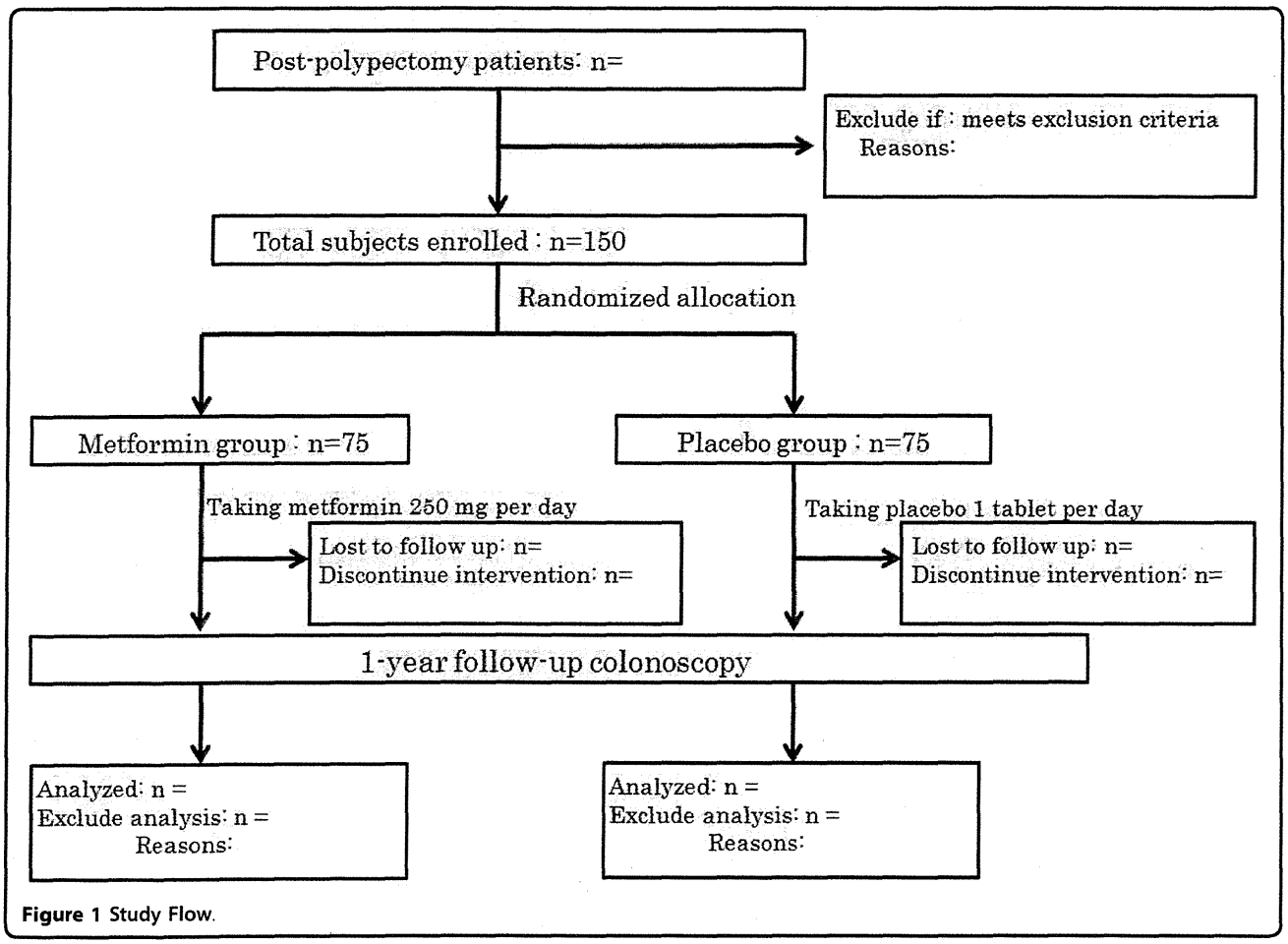

Figure 1 Study Flow.

the formation of human colorectal ACF and was also safe, leading us to surmise that oral low-dose metformin may also be effective for CRC chemoprevention. Therefore, we planned to conduct this trial with low-dose metformin. Second, repeat colonoscopy at 1 year may be too short to allow reliable detection of differences between the groups. However, this is first chemoprevention study of metformin for CRC, long-term administration of placebo to post-polypectomy patients may entail ethical problems. Therefore, this time we planned repeat colonoscopy at 1 year. In order to detect the effect of metformin with less effort, we elected to select participants who had undergone polypectomy for this trial, because these patients constitute a high-risk group for the development of metachronous colorectal polyps and/or CRC [5]. And after safety of chronic metformin administration for non-diabetic patients is confirmed, we would like to follow up the participants of this trial, and conduct long-term chemoprevention trial for CRC.

If metformin were found to be effective for the prevention of CRC, the impact would be extremely large. We consider it of interest, therefore, to determine whether metformin might suppress the formation of human colorectal polyps.

Abbreviations

CRC: Colorectal cancer; NSAIDs: Nonsteroidal anti-inflammatory drugs; COX-2: Cyclooxygenase-2; ACF: Aberrant crypt foci; AMPK: AMP-activated protein kinase; PCNA: Proliferative cell nuclear antigen; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

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Current study status

This trial began recruiting patients in September 2011 and shall close to recruitment in December 2012. Data collection is due to be completed in December 2013 and results will be published in March 2014.

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Authors' contributions

TH and AN conceived the study. KH conducted feasibility phase work. HT, TU, NH, US, HK and NM shall perform polypectomy and follow-up colonoscopy. HE, HO and ES will make another count of polyps on DVD record to ensure validity. EY, NH, YH, AE, HN and NM shall recruit participants and follow-up at outpatient clinic. Analysis and interpretation of data is being conducted by SM. SY and YI shall carry out pathological analysis. All authors have read and approve of the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Eicosapentaenoic acid (EPA) efficacy for colorectal aberrant crypt foci (ACF): a double-blind randomized controlled trial

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Abstract

Background: Colorectal cancer (CRC) is one of the most commonly occurring neoplasms and a leading cause of cancer death worldwide, and new preventive strategies are needed to lower the burden of this disease. Eicosapentaenoic acid (EPA), the omega-3 polyunsaturated fatty acid that is widely used in the treatment of hyperlipidemia and prevention of cardiovascular disease, has recently been suggested to have a suppressive effect on tumorigenesis and cancer cell growth. In CRC chemoprevention trials, in general, the incidence of polyps or of the cancer itself is set as the study endpoint. Although the incidence rate of CRC would be the most reliable endpoint, use of this endpoint would be unsuitable for chemoprevention trials, because of the relatively low occurrence rate of CRC in the general population and the long-term observation period that it would necessitate. Moreover, there is an ethical problem in conducting long-term trials to determine whether a test drug might be effective or harmful. Aberrant crypt foci (ACF), defined as lesions containing crypts that are larger in diameter and stain more darkly with methylene blue than normal crypts, are considered as a reliable surrogate biomarker of CRC. Thus, we devised a prospective randomized controlled trial as a preliminary study prior to a CRC chemoprevention trial to evaluate the chemopreventive effect of EPA against colorectal ACF formation and the safety of this drug, in patients scheduled for polypectomy.

Methods: This study is a multicenter, double-blind, placebo-controlled, randomized controlled trial to be conducted in patients with both colorectal ACF and colorectal polyps scheduled for polypectomy. Eligible patients shall be recruited for the study and the number of ACF in the rectum counted at the baseline colonoscopy. Then, the participants shall be allocated randomly to either one of two groups, the EPA group and the placebo group. Patients in the EPA group shall receive oral 900-mg EPA capsules thrice daily (total daily dose, 2.7 g per day), and those in the placebo group shall receive oral placebo capsules thrice daily. After one month's treatment with EPA/placebo, colonoscopic examination and polypectomy will be performed to evaluate the formation of ACF, and the cell-proliferative activity and cell-apoptotic activity in normal colorectal mucosa and colorectal polyps.

Discussion: This is the first study proposed to explore the effect of EPA against colorectal ACF formation in humans.

This trial has been registered in the University hospital Medical Information Network (UMIN) Clinical Trials Registry as UMIN000008172.

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Background

Colorectal cancer (CRC) is amongst the most commonly encountered neoplasms worldwide [1], and both its prevalence and mortality have been increasing [2]. Removal of colorectal polyps has been to shown to reduce the risk of future development of colorectal cancer and advanced adenoma [3,4] and to thereby prevent colorectal cancer death [5]. On the other hand, patients with polyps (adenomas and/or hyperplastic polyps) also constitute a high-risk group for the development of metachronous colorectal polyps and/or CRC [6]. Therefore, a paradigm shift from surveillance for early detection of cancer or adenomas and polypectomy to new strategies for prevention, including chemoprevention, is needed to lower the burden of this disease. Several large epidemiologic and/or clinical studies have evaluated the possible effects of more than 200 agents, including fiber, calcium, and non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin and selective cyclooxygenase-2 (COX-2) inhibitors, in protecting against CRC development [7]. Our group previously reported that sulindac, a NSAID, had the effect of suppressing the development of sporadic colorectal adenoma [8]. Until date, NSAIDs, especially COX-2 inhibitors, administered either alone or in combination with other agents, have shown the most promise for CRC risk reduction [4], although reports have revealed an increased risk of serious cardiovascular events associated with the use of COX-2 inhibitors [9,10]. In light of the adverse cardiovascular effects of COX-2 inhibitors and the lack of demonstrable efficacy of the other agents that had initially shown promise in this setting, novel drugs that would be both safe and effective for CRC prevention need to be developed. CRC is known to be associated with lifestyle-related diseases, such as hyperlipidemia, diabetes mellitus and obesity [11-14], therefore, we considered that these conditions might represent potential new targets for CRC chemoprevention.

Eicosapentaenoic acid (EPA) is an omega-3 polyunsaturated fatty acid (PUFA) that has long been used widely for primary and also secondary prevention of cardiovascular diseases [15]. EPA impacts the biological functions of adipocytes via two distinct mechanisms; the first, via transcriptional activation of lipogenic and adipogenic genes by binding to nuclear receptors such as Peroxisome Proliferator Activator Receptors (PPARs),[16] and the second, via direct competition with arachidonic acid (AA) incorporation into membrane phospholipids and subsequent conversion to eicosanoids, including prostaglandins (PGs). [17] Recent reports have indicated a lower incidence of colon, breast and prostate cancers in many human populations, associated with a high dietary consumption of omega-3 PUFAs. Multiple reports using a variety of rodent models of early-stage colorectal carcinogenesis, including azoxymethane- and dimethylhydrazine-induced colorectal tumorigenesis (using aberrant crypt foci (ACF) or colonic tumors as the primary endpoint), as well as the $Apc^{Min/+}$ mouse model of familial adenomatous polyposis (FAP), have demonstrated the efficacy of the free fatty acid (FFA) form of a combination of EPA plus docosahexaenoic acid (DHA) (as fish oil substituted for the base fat source in chow).[18] In humans, a phase-III randomized placebo-controlled trial of EPA-FFA 2 g daily for 6 months was performed in 55 patients with FAP undergoing sigmoidoscopic surveillance of a rectal stump after total colrectomy. [19] Patients in the EPA-FFA arm had a significantly lower (by 22.4%) number of lower rectal polyps and a 29.8% decrease in the sum of the polyp diameters in the tattooed area of the rectum as compared with the placebo group. Importantly, daily administration of EPA-FFA 2 g was safe and well-tolerated. [19] NSAIDs chemoprevention trial set in past, first conducted to FAP patients, then applied to sporadic colorectal adenoma/cancer. Thus, much evidence suggests that EPA might be a candidate agent for CRC chemoprevention. In CRC chemoprevention trials, in general, the incidence of polyps or of the cancer itself is set as the study endpoint. Although the incidence rate of CRC would be the most reliable endpoint, use of this endpoint would be unsuitable for chemoprevention trials, because of the relatively low occurrence rate of CRC in the general population [20] and the long-term observation period that it would necessitate. Moreover, there is an ethical problem in conducting a long-term trial to determine whether a test drug may be effective or harmful.

Aberrant crypt foci (ACF), defined as lesions containing crypts that are larger in diameter and stain more darkly with methylene blue than normal crypts,[21-24] are considered as a reliable surrogate biomarker of CRC. [25] We previously reported the usefulness of ACF as a biological marker of CRC, [26,27] and carried out a chemoprevention trial for colorectal ACF. [28,29] Chemoprevention trials with colorectal ACF set as the primary endpoint may have some advantages. First, a long-term observation period is not needed to evaluate the drug effect. Our group reported the n.[29] Long-term trials need much effort and may expose the study participants to an increased risk of development of carcinoma. Second, ACF can be estimated quantitatively. Thus, we devised a prospective randomized controlled trial to evaluate the chemopreventive effect of EPA against the formation of colorectal ACF as a preliminary study prior to CRC chemoprevention trials.

This is the first clinical trial of EPA as a chemopreventive agent against colorectal ACF in humans.

Methods/design

Study design and setting

This study is designed as a multicenter, double-blind, placebo-controlled, randomized controlled trial to be

performed in patients with colorectal ACF. It will be conducted at the Division of Gastroenterology, Yokohama City University Hospital, and its affiliate hospital, Chigasaki Municipal Hospital and Yokohama Rosai Hospital. The coordinating office shall be at the Yokohama City University Hospital, and the registration, randomized allocation and data collection shall be conducted at this site.

Ethical considerations and registration

The study protocol is in compliance with the Declaration of Helsinki [30] and the Ethics Guidelines for Clinical Research published by the Ministry of Health, Labour, and Welfare, Japan [31]. We obtained approval for this study from the Ethics committee of Yokohama City University Hospital on May 10, 2012. The protocol and informed consent forms were approved by the institutional ethics committee at each of the participating institutions. This trial has been registered in the University hospital Medical Information Network (UMIN) Clinical Trials Registry as UMIN000008172. Written informed consent for participation in the study will be obtained from all the participating patients. The trial results will be reported in conformity with the Consolidated Standards of Reporting Trials (CONSORT) 2010 guidelines [32].

Eligibility criteria

Patients with both colorectal ACF and resectable polyps will be recruited for the study.

The proposed inclusion criteria are as follows:

1) Age 40 to 80 years as on the date of informed consent.
2) Willingness to provide written informed consent.

The proposed exclusion criteria are as follows:

1) History of regular use of omega-3 PUFA supplements.
2) History of regular use (defined as at least once per week) of NSAIDs and/or aspirin.
3) History of heart failure, renal failure, liver cirrhosis or chronic hepatic failure.
4) History of familial adenomatous polyposis.
5) History of hereditary non-polyposis colorectal cancer.
6) History of inflammatory bowel disease.
7) Pregnancy or possibility of pregnancy.
8) Patients judged as being inappropriate candidates for the trial by the investigators.

Intervention

All eligible patients will be allocated randomly to one of two groups, the EPA group and the placebo group. The endoscopists, doctors at the follow-up outpatient clinics, and patients will be blinded to the allocation. Patients in the EPA group shall receive oral 900-mg EPA capsules thrice daily (total daily dose, 2.7 g), and those in the placebo group shall receive oral placebo capsules thrice a day. At the end of 1 month of administration of EPA/placebo, polypectomy will be performed, and the changes in the number of ACF and in the mucosa will be evaluated.

Outcome measurements

The primary endpoint shall be the change in the number of colorectal ACF after 1-months's intervention. The endoscopic examinations and polypectomies will be performed using Olympus colonoscopes (model H260AZI). Bowel preparation prior to the colonoscopic procedures will be as described [33,34]. At the time of the first colonoscopy, the endoscope shall be inserted into the cecum, and the entire colorectum will be carefully observed as the endoscope is pulled back. If any polyps are detected, biopsy will be performed. Furthermore, colonic epithelial samples will be obtained. The number of rectal ACF will be counted with a magnifying endoscope, as described [25,33]. At the end of 1 month of administration of EPA/placebo, the same endoscopists will perform the polypectomy and counting of the ACF. All procedures will be recorded on DVD, and all the ACF will be photographed. The number of ACF in each patient will first be counted by the operators during the performance of the colonoscopy. To further ensure validity, the number of ACF will be counted again through observation of the recorded DVD by 3 blinded expert endoscopists (H.T, H.E, and E.S). If these expert endoscopists judge the colonoscopic examination as having been inadequate in any case, that case will be excluded.

The secondary outcomes shall be (1) the drug safety; adverse events (AEs) will be graded according to the National Cancer Institute Common Toxicity Criteria for Adverse Events (NCI-CTCAE), version 4.0. All study participants shall be provided with a study diary in order to record the daily dosage of the study treatment and the AEs. Patients developing grade 3 or more severe adverse events will be withdrawn from the study at that point; (2) Mucosal fatty acid analysis: Homogenization, extraction and derivatization of the rectal mucosa and polyp fatty acids (EPA, DHA, docosapentaenoic acid (DPA), AA, linolenic acid, linoleic acid, palmitic acid, stearic acid, etc.) shall be performed as described [35]. Fatty acid content shall be determined by gas chromatography–mass spectrometry and expressed as the percentage of the total fatty acid content [36,37]. (3) Effects of EPA on the cell-proliferative and apoptotic activities in the rectal epithelium and polyps: Colonic epithelial samples will be obtained from the same trial patients by biopsy at the time of the first colonoscopy and

polypectomy. The cell-proliferative activity will be evaluated by staining for the proliferative cell nuclear antigen (PCNA) and estimation of the Ki-67 labeling indices, and the cell-apoptotic activity by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) method. (4) Laboratory data (HDL cholesterol, LDL cholesterol, triglycerides, fatty acid fractions, fasting blood glucose, fasting blood insulin, HbA1c, blood urea nitrogen (BUN), creatinine); (5) physical examination findings (body weight, body mass index (BMI)). EPA is widely used as an anti-hyperlipidemic drug that improves the plasma lipid profile. The effect of EPA on the plasma lipid profile will be evaluated by comparing these parameters measured at the baseline with those measured after 1 month of treatment in the EPA group and placebo group. All participants will undergo a physical examination and laboratory tests at the time of the baseline endoscopic examination and polypectomy.

Randomization

The investigator shall convey the patient's details to the central registration center via fax. After an eligibility check, the patients will be randomly assigned to receive EPA or placebo at the central registration center by a computer program using a minimization method, with stratification by age, gender, BMI, and institution. Thus, the patient assignment will be concealed from the investigator. The randomization center will allocate a numbered treatment pack to each patient, which will contain all the drugs or placebos needed to complete a course of the trial treatment for that patient.

Drug supply

Enteric-coated EPA capsules (Ethyl icosapentate granular capsule®) and the placebo capsules (capric, caprylic and lauric acid medium-chain triglycerides) will be purchased from Nipro Pharma Corporation Co., Ltd, Osaka, Japan. All trial drugs will be packaged identically and identified only by number. Subjects will be instructed to take one package of the trial drug after every meal each day. Compliance will be monitored by counting the empty drug packages returned by the patients at polypectomy.

Sample size estimation

In the chemoprevention trial conducted in FAP patients, the NSAID sulindac and selective cyclooxygenase-2 (COX-2) inhibitor celecoxib reduced polyposis of the retained rectum after colectomy with ileorectal anastamosis (IRA). As previously noted, EPA has a suppressive effect for polyp formation and proliferation of FAP [19]. From these reports, we estimated that NSAIDs and EPA may have equivalent effect on suppression of polyp formation and proliferation.

Based on the target in the NSAIDs chemoprevention study for ACF, Takayama et al. reported that sulindac administration at 300 mg/d for 2 months to post-polypectomy patients suppressed ACF formation, decreasing the number of ACF from 7.70 ± 4.04 (baseline) to 4.00 ± 2.95 (at 2 months, $p < 0.001$) [33]. Presuming EPA and sulindac may have equivalent effect in suppressing ACF formation, to detect the reduction in the number of ACFs in the EPA group using the Mann–Whitney U test with a two-sided significance level of 5% and a power of 80%, it was estimated that a sample size of 12 patients per group would be necessary. Assuming a 10% dropout rate, we propose to recruit 15 patients per group, that is, a total of 30 patients.

Statistical analysis

The number of ACFs in each group, the primary endpoint, will be compared between the EPA group and the placebo group by the Mann–Whitney U test. The safety, one of the secondary endpoints, will be compared by the chi-square test. The remaining results in the two groups will be compared by the Mann–Whitney U test or Student's t test. A P values of < 0.05 will be regarded as indicative of significance. The analysis will be performed using SPSS, version 17.0 (SPSS Inc., Chicago, Il.).

Trial Steering Committee and Data Monitoring Committee

The Trial Steering Committee and Data Monitoring Committee shall be located at the Department of Gastroenterology, Kanto Medical Center, NTT East. The committee shall consist of three people: Nobuyuki Matsuhashi, M.D., Toshio Fujisawa, M.D., and Jun Hamanaka, M.D. The Management Team will monitor the trial progress status and data by face-to-face and/or telephonic contact with each of the sites every month.

Study flow

A flow chart of the study is shown in Figure 1.

Discussion

This is the first study proposed to explore the effect of EPA against colorectal ACF formation. Knowledge of the mechanisms underlying the anti-neoplastic activity of EPA remains nebulous. Current understanding of the mechanistic aspects of the anticancer activity of EPA has been reviewed in detail in published reviews [38-41]. In general, the major mechanisms proposed to underlie the anti-neoplastic activities of EPA; (1) modulation of COX activity, (2) alteration of the membrane dynamics and cell surface receptor function, and (3) increased cellular oxidative stress. However, the in vivo relevance of each of the above putative mechanisms and their relative

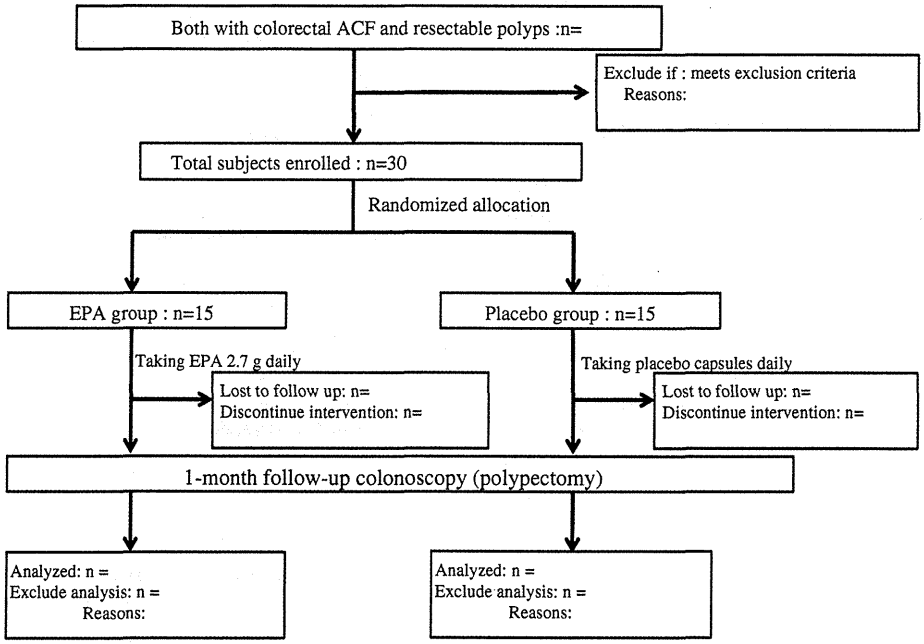

Figure 1 Study Flow.

contributions to the anticancer activity of EPA remain unclear. Several in-vitro studies have explored the antineoplastic activity of omega-3 PUFAs against human CRC cells, and EPA treatment has been shown to reduce cellular proliferative activity and increase cellular apoptotic activity [42-45]. EPA can act as an alternative substrate for COX-2, instead of AA, leading to a reduction in the formation of the pro-tumorigenic '2-series' PGs (e.g., PGE_2) in favor of the 'three-series' PGs (e.g., PGE_3) in several cell types, including CRC cells [46-48]. Furthermore, incorporation of EPA into the cell phospholipid membrane alters the fluidity, structure, and/or function of the lipid rafts or calveolae [49], which are sphingolipid- and cholesterol-rich microdomains that float freely in the cell membrane. The localization of cell surface receptors, such as epidermal growth factor receptor (EGFR) [50], in lipid rafts is believed to be crucial for downstream receptor signaling, controlling proliferation and apoptosis [51,52]. Furthermore, EPA may exert an antineoplastic effect through alteration of the cellular redox state and of the oxidative stress exposure of the cells. PUFAs are highly peroxidizable, which generates reactive oxygen species (ROS), such as superoxide radicals. Many tumor cells display altered cellular pathways for the handling of ROS, including depletion of the major intracellular antioxidant, glutathione. Subsequent elevation of the intracellular ROS levels by EPA has been hypothesized to induce cancer cell apoptosis [53].

This trial may have the following limitations. First, ACF are considered as a reliable surrogate biomarker of CRC, [21] although their biological significance still remains controversial. In CRC chemoprevention trials, in general, the incidence of polyps or of the cancer itself is set as the study endpoint. Although the incidence rate of CRC would be the most reliable endpoint, use of this endpoint would be unsuitable for chemoprevention trials, because of the relatively low occurrence rate of CRC in the general population [18] and the long-term observation period that it would necessitate. We previously reported the usefulness of ACF as a biological marker of CRC [26,27] and carried out a chemoprevention trial for colorectal ACF [28,29]. Thus, we devised a trial using this endpoint (ACF) to evaluate the chemopreventive effect of EPA. Second, we do not propose to conduct a dose–response study in respect of the effect of EPA on ACF formation. Until now, trials of EPA for cancer prevention and adjuvant treatment have been conducted using EPA at doses of 1000 – 4000 mg per day. In Japan, the EPA drug product specification is 0.9 g, and 2.7 g of EPA has been commonly used and very well tolerated. Therefore, we planned to conduct this trial using 2.7 g of EPA per day. Third, an intervention period of 1 month may be too short to allow reliable detection of differences between the groups. However, we showed in a previous study that oral administration of metformin for 1 month suppressed the formation of colorectal ACF in humans [29]. If the intervention agents had a chemopreventive effect, an intervention period of 1 month would be sufficient to evaluate the changes in the number of ACF.

We previously conducted a short-term chemoprevention trial of metformin for colorectal ACF, and showed

the suppressive effect of the drug on the formation of ACF. Thereafter, we are conducting a long-term metformin chemoprevention trial for colorectal polyps, the trial registered in the UMIN Clinical Trials Registry as UMIN000006254 [34]. We propose to repeat the same step for the chemoprevention trial using EPA.

If EPA were found to be effective for the prevention of CRC, the impact would be extremely large. We consider it of interest, therefore, to determine whether EPA might suppress the formation of human colorectal ACFs.

Abbreviations

CRC: Colorectal cancer; NSAIDs: Nonsteroidal anti-inflammatory drugs; COX-2: Cyclooxygenase-2; ACF: Aberrant crypt foci; EPA: Eicosapentaenoic acid; PUFA: Polyunsaturated fatty acid; AA: Arachidonic acid; PG: Prostaglandin; FAP: Familial adenomatous polyposis; FFA: Free fatty acid (FFA); DHA: Docosahexaenoic acid; PCNA: Proliferative cell nuclear antigen; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling.

Competing interests

None of the authors has any financial interests relevant to this trial to disclose.

Authors' contributions

TH and AN conceived the study. HT, TU and AE shall perform the baseline colonoscopy and polypectomy. HE, HO and ES will conduct another count of ACF on a DVD recording to ensure its validity. HI, SU, SM and HN shall recruit participants and follow-up at outpatient clinic. EY and KH shall carry out the pathological analyses. Analysis and interpretation of data will be conducted by YN and SM. All the authors have read the final manuscript and approve of its submission for publication.

Current study status

This trial began recruiting patients in June 2012 and shall complete recruitment in December 2012. Data collection is due to be completed in March 2013, and the results are scheduled to be published in June 2013.

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Arf and p53 act as guardians of a quiescent cellular state by protecting against immortalization of cells with stable genomes

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ABSTRACT

Normal cells undergo a growth-arrested status that is produced by p53-dependent down-regulation of histone H2AX. Immortality is developed after abrogation of the H2AX-diminished state, which is associated with genomic instability (often with tetraploidy) and the induction of mutations in either the *Arf* or *p53* gene. However, the role of Arf in control of H2AX expression and genome stability is still unclear. Here, we show that both Arf and p53 are required for the down-regulation of H2AX and formation of the growth-arrested state. Wild-type (WT) mouse embryonic fibroblasts (MEFs) subjected to tetraploidization with DNA lesions did not undergo mitotic catastrophe-associated cell death and stayed in a growth-arrested state, until immortality was attained with mutations in the *Arf/p53* module and recovery of H2AX expression. Whereas tetraploidization was essential for immortalization of WT MEFs, this event was not required for immortalization of MEFs containing mutations in *Arf/p53* and these cells still underwent mitotic catastrophe-associated cell death. Thus, WT MEFs are protected from immortalization with genome stability, which is abrogated with tetraploidization and mutation of either *Arf* or *p53*.

1. Introduction

Most cancers that develop in old age are characterized by chromosomal or microsatellite instabilities, as well as mutations in genes, such as those involved in the Arf-MDM2-p53 axis [1–3]. Similar to cancer cells, mouse embryonic fibroblasts (MEFs) acquire immortality associated with genomic instability [4] and mutations in the *Arf/p53* module [5]. Although *Arf* and *p53* are part of the same regulatory module, these genes are mutated in a mutually exclusive manner in immortalized MEFs, suggesting that both Arf and p53 are required for protection against cellular immortalization [6,7]. By contrast, the p53-dependent acute response to damage still occurs in p53-proficient cancer cells that contain mutations in *Arf* [8,9]. These findings suggest that normal cells are protected from immortalization by regulation of both Arf and p53, and that this protection mechanism is distinct from the role of p53 in the acute damage response [6].

Because Arf and p53 are critical tumor suppressors, *Arf*- and *p53*-knockout (KO) mice are predisposed to cancer development [10,11]. In addition, transgenic mice with an extra copy of the *Arf* and *p53* genes (super-*Arf/p53* mice) show signs of cancer suppression and have extended life spans [5]. Intriguingly, like wild-type MEFs with stable genomes, MEFs from super-*Arf/p53* mice are strongly protected against immortalization [5]. These findings imply that the primary function of the Arf/p53 module is control of cellular homeostasis, which contributes to lifespan extension and cancer suppression. By contrast, cells with hyperactive p53 induced by overexpression or acute damage undergo senescence or apoptosis *in vitro* [12–14], and transgenic mice with hyperactive p53 undergo premature aging [15–17]. Furthermore, mutant mice that are unable to induce many of the canonical p53 target genes in response to acute DNA damage retain tumor suppression activity under normal conditions [8,9]. Taken together, these findings suggest that p53 has distinct functions under normal and hyperactivated conditions; the Arf-dependent function of p53 is to control cellular homeostasis under normal conditions, leading to lifespan extension and cancer prevention, and is likely to be distinct from the function of hyperactivated p53 [6].

After serial proliferation, normal cells generally undergo a growth-arrested state associated with diminished levels of H2AX

Abbreviations: CTU, camptothecin; HU, hydroxyurea; KO, knockout; MEFs, mouse embryonic fibroblasts; WT, wild-type.

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[6,7]. These growth-arrested cells are defective in DNA damage repair and are therefore susceptible to the accumulation of unrepaired DNA lesions [18]. In response to aberrantly accelerated growth stimuli, these growth-arrested cells develop DNA replication stress-associated lesions and subsequent genomic instability [4]. Cellular growth retardation and DNA damage repair deficiency are both likely caused by a reduction in histone H2AX levels because cells lacking H2AX also display these characteristics [19–22]. By contrast, transformed or immortalized cells are formed following abrogation of the H2AX-diminished state [6,7].

Down-regulation of H2AX is dependent on p53; the mechanism of regulation presumably involves the Arf/p53 module because the H2AX-diminished and growth-arrested state is not induced in *p53*-KO MEFs or in immortalized MEFs that contain mutations in either *Arf* or *p53* [6,7]. Although the role of p53 in establishment of a quiescent state has been described previously [6,7], the mechanism by which Arf contributes to the down-regulation of H2AX, protection against immortalization, and genomic instability (ploidy) is still unclear.

In this study, we demonstrate that Arf is required for growth arrest associated with reduced levels of H2AX in MEFs. The quiescent state of normal MEFs was abrogated by mutations in either *Arf* or *p53*. Although tetraploidization was not essential for immortalization of *p53*-KO and *Arf*-KO MEFs, tetraploidization of wild-type (WT) MEFs was required to induce mutations in the Arf/p53 module.

2. Materials and methods

2.1. Cell culture

WT, *Arf*-KO, and *p53*-KO MEFs were prepared from Day 13.5 mouse embryos, as previously described [7]. MEFs were cultured as described previously [23] and were passaged using the standard 3T3 protocol [24], unless otherwise indicated. DNA replication stress-associated damage was induced by the treatment of cells with camptothecin (CPT) (Sigma) or hydroxyurea (HU) (Sigma) as indicated in each figure.

2.2. Antibodies and immunoblotting

Antibodies against H2AX (Bethyl Laboratories), γH2AX (Millipore-Upstate), β-actin (AC-74, Sigma), Parp1 (Cell Signaling Technology), cleaved caspase-3 (Cell Signaling Technology), and histone H3 (MABI0301, Monoclonal Antibody Institute) were used in this study. Immunoblotting was performed as described previously [23].

2.3. Analyses of the chromosomal status

For analyses of mitotic phase chromosomes, cells were treated with 200 ng/ml nocodazole for 5 h and then mitotic cells were collected. The cells were hypotonically swollen by treatment with 75 mM KCl for 30 min, and then fixed with Carnoy's solution (60% methanol, 30% acetic acid, and 10% chloroform) for 20 min. After changing the fixative once, cells were dropped onto glass slides and air-dried [4]. The slides were stained with 4% Giemsa stain (Merck) for 10 min, washed briefly in tap water, and then air-dried. For FACS analyses of the cellular ploidy status, harvested cells were incubated in PBS containing RNase A (200 μg/ml, Sigma) for 30 min on ice and then stained with propidium iodide (20 μg/ml, Sigma) for an additional 30 min on ice in the dark. The stained cells were analyzed by flow cytometry (Beckman Coulter).

3. Results

3.1. Arf-KO MEFs do not undergo H2AX-diminished growth arrest

To determine the role of Arf in the establishment of a H2AX-diminished and growth-arrested state, experiments were performed using primary WT and *Arf*-KO MEFs. Unlike WT MEFs, the *Arf*-KO MEFs did not undergo growth arrest and continued to

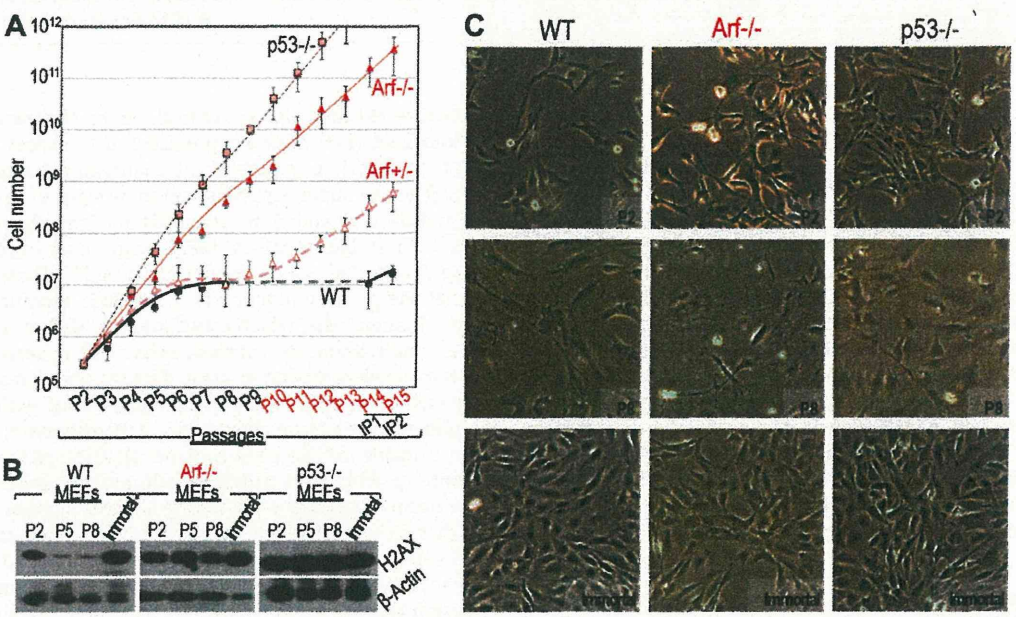

Fig. 1. Arf-KO MEFs do not undergo H2AX-diminished growth arrest. (A) Growth curves of MEFs (WT, $Arf^{+/-}$, $Arf^{-/-}$, and $p53^{-/-}$) cultured under a standard 3T3 passage protocol. Unlike WT and $Arf^{+/-}$ MEFs, $Arf^{-/-}$ MEFs continuously grew and developed immortality. Data show the mean ± SD of $n = 3$ independent experiments. IP1 and IP2 indicate Immortal Passage 1 and 2 for WT MEFs. (B) Immunoblot analysis of histone H2AX expression in passage 2 (P2), P5, P8, and immortalized MEFs (WT, $Arf^{-/-}$, and $p53^{-/-}$). Expression levels of β-actin were used as a loading control. Unlike WT MEFs, $Arf^{-/-}$ and $p53^{-/-}$ MEFs failed to form the H2AX-diminished state. (C) Morphologies of P2, P8, and immortalized WT, $Arf^{-/-}$, and $p53^{-/-}$ MEFs. Similar to WT MEFs, $Arf^{-/-}$ and $p53^{-/-}$ MEFs displayed a senescent morphology before acquiring the immortalized morphology.

immortalize across 15 passages (Fig. 1A); this result is consistent with a previous report [11] and with the growth of *p53*-KO MEFs. In addition, immunoblot analyses revealed that expression levels of H2AX in the *Arf*-KO MEFs and *p53*-KO MEFs were not down-regulated across multiple passages (Fig. 1B). These data support the notion that the establishment of an H2AX-diminished and growth-arrested state in normal cells is regulated by both Arf and p53, and that this state is abrogated by genomic instability caused by mutations in the Arf/p53 module.

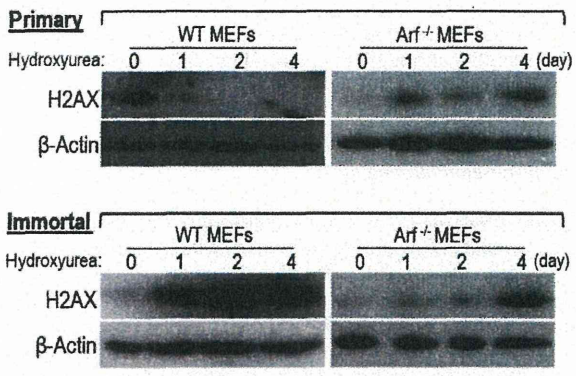

Fig. 2. DNA replication stress-induced down-regulation of H2AX is dependent on Arf. DNA replication stress was induced in primary and immortalized WT and *Arf*$^{-/-}$ MEFs by treatment with 0.2 mM HU for up to 4 days. The DNA replication stress-induced down-regulation of H2AX was abrogated in immortalized WT MEFs and in both primary and immortalized *Arf*$^{-/-}$ MEFs.

In spite of the lack of growth arrest, the flattened and enlarged cellular morphology of both *p53*-KO and *Arf*-KO MEFs was similar to that of the senescent WT MEFs. The immortalized KO cells subsequently acquired the morphology typically seen in immortalized MEFs (Fig. 1C). This result indicates that both Arf and p53 are required for the establishment of the H2AX-diminished state but are not essential for the formation of some of the typical senescent characteristics of the cells, including the flattened and enlarged morphology. In addition, since growth retardation is observed following knockdown of H2AX [7,19], Arf- and p53-dependent diminution of H2AX is likely a direct cause of the growth-arrested state of normal cells.

To examine the effect of Arf on the down-regulation of H2AX directly, DNA replication stress was induced in WT and *Arf*-KO MEFs by exposing cells to HU that depletes dNTP pool. The expression level of H2AX in *Arf*-KO MEFs was compared with that in WT MEFs because H2AX is down-regulated during DNA replication stress in WT MEFs but not in *p53*-KO MEFs. As expected, H2AX expression was down-regulated in primary WT MEFs after 1–4 days of treatment with HU. By contrast, H2AX expression was not down-regulated in immortalized WT MEFs, primary *Arf*-KO MEFs, or immortalized *Arf*-KO MEFs (Fig. 2); this result agrees with a previous report of stable H2AX expression in DNA replication stress-induced *p53*-KO MEFs [7]. These data further support the proposal

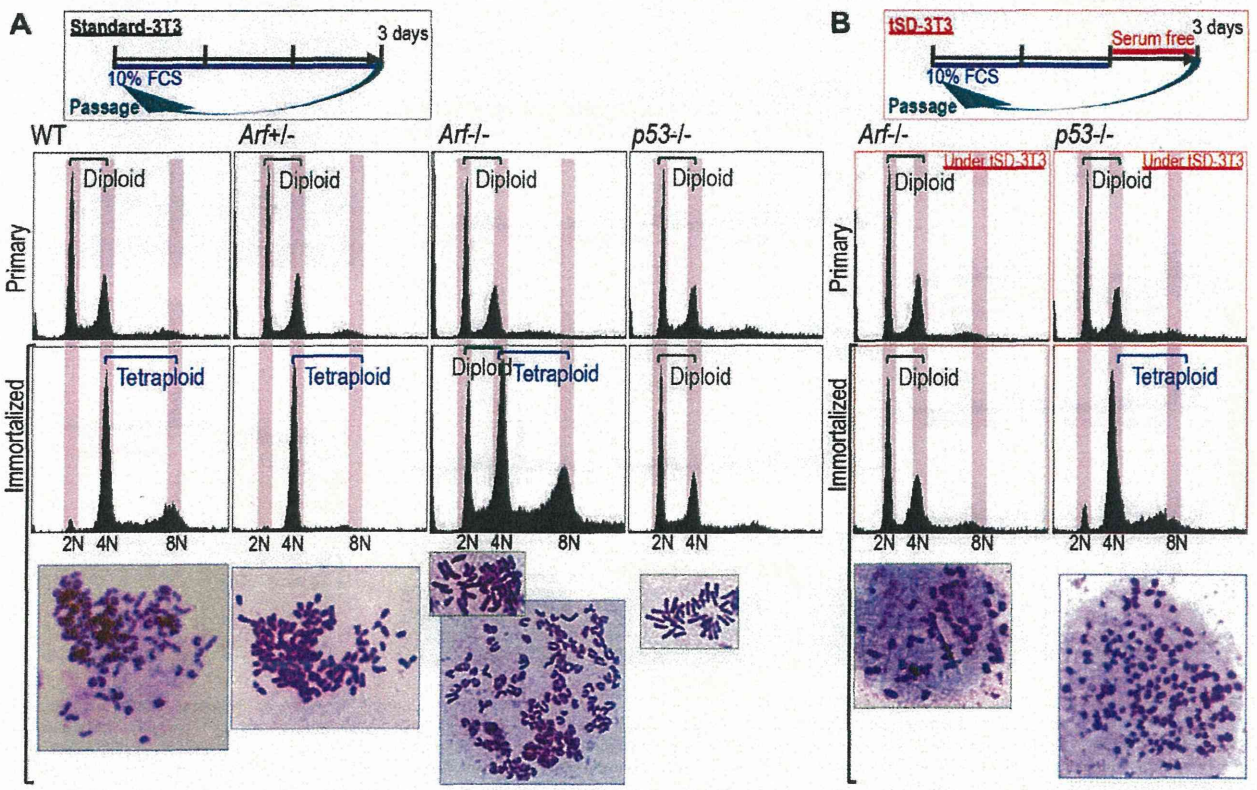

Fig. 3. Tetraploidization is not essential for immortalization of *Arf*$^{-/-}$ and *p53*$^{-/-}$ MEFs. (A) FACS analyses of changes in the cellular ploidy status of primary and immortalized WT, *Arf*$^{+/-}$, *Arf*$^{-/-}$, and *p53*$^{-/-}$ MEFs cultured under standard 3T3 conditions. WT and *Arf*$^{+/-}$ MEFs showed tetraploidization after development of immortality, while *p53*$^{-/-}$ MEFs immortalized with diploidy. Immortalized *Arf*$^{-/-}$ MEFs were a mixture of dipoid and tetraploid states. Giemsa stains of M-phase chromosomes are also shown. Images are the representatives (diploidy with green frame and tetraploidy with blue frame). (B) FACS analyses of changes in the cellular ploidy status of *Arf*$^{-/-}$ and *p53*$^{-/-}$ MEFs during culture under growth-restricted tSD-3T3 conditions. Under these conditions, *Arf*$^{-/-}$ MEFs immortalized with diploidy, whereas immortalized *p53*$^{-/-}$ MEFs developed tetraploidy. Giemsa stains of M-phase chromosomes are also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that the H2AX-diminished and growth-arrested state of normal cells is dependent on both Arf and p53, and that this quiescent state is abrogated by knockout of the Arf/p53 module, which causes recovery of H2AX expression and subsequent growth activity.

3.2. Immortalized Arf-KO MEFs are a mixture of diploid and tetraploid cells

In WT MEFs, mutation of the Arf/p53 module is induced during tetraploidization and leads to the recovery of H2AX expression and development of immortality [7]. By contrast, *p53*-KO MEFs maintain a stable diploid state even after immortalization [7]. These previous observations motivated us to examine the genomic status of *Arf*-KO MEFs during immortalization. FACS analysis revealed that immortalized *Arf*-KO MEFs were a mixture of tetraploid and diploid states (Fig. 3A). Giemsa staining also showed that the M-phase chromosomes of immortalized *Arf*-KO MEFs were a mixture of two types: one similar to the chromosomes of immortalized WT MEFs and another similar to those of immortalized *p53*-KO MEFs (Fig. 3A). Since *p53*-KO and *Arf*-KO MEFs acquired immortality with diploidy (at least partly in the case of *Arf*-KO MEFs), these findings suggest that tetraploidization is not essential for immortalization in an *Arf*-mutated or *p53*-mutated background.

To address whether the tetraploidization of *Arf*-KO MEFs is induced in a similar manner to that of WT MEFs, *Arf*-KO MEFs were continuously cultured under a 3T3 passage protocol with temporary depletion of serum for the day immediately prior to passage (tSD-3T3). Under these conditions, tetraploidization of immortalized WT MEFs is neutralized and cells are continuously growth arrested with stable diploidy [7]. Tetraploidization of immortalized *Arf*-KO MEFs was also inhibited when cells were cultivated under the tSD-3T3 conditions (Fig. 3B); this result confirms the hypothesis that tetraploidization is not required for immortalization in an *Arf*-mutated background. In addition, these results indicate that MEFs lacking Arf develop tetraploidy in response to accelerated growth stimuli, although this tetraploidization does not affect growth activity or immortalization.

3.3. Immortalized p53-KO MEFs cultured under growth-restricted conditions develop tetraploidy

Under standard 3T3 culture conditions, *p53*-KO MEFs maintained a diploid status during immortalization (Fig. 3A); however, these cells developed tetraploidy under growth-restricted (tSD-3T3) conditions (Fig. 3B). This result was unexpected because tetraploidization of the *Arf*-KO MEFs was inhibited under the tSD-3T3 conditions. Although the mechanism by which tetraploidization is induced in *p53*-KO MEFs is unclear, this event may be associated with oxidative stress because the level of reactive oxygen species is elevated during serum depletion [25,26]. Although

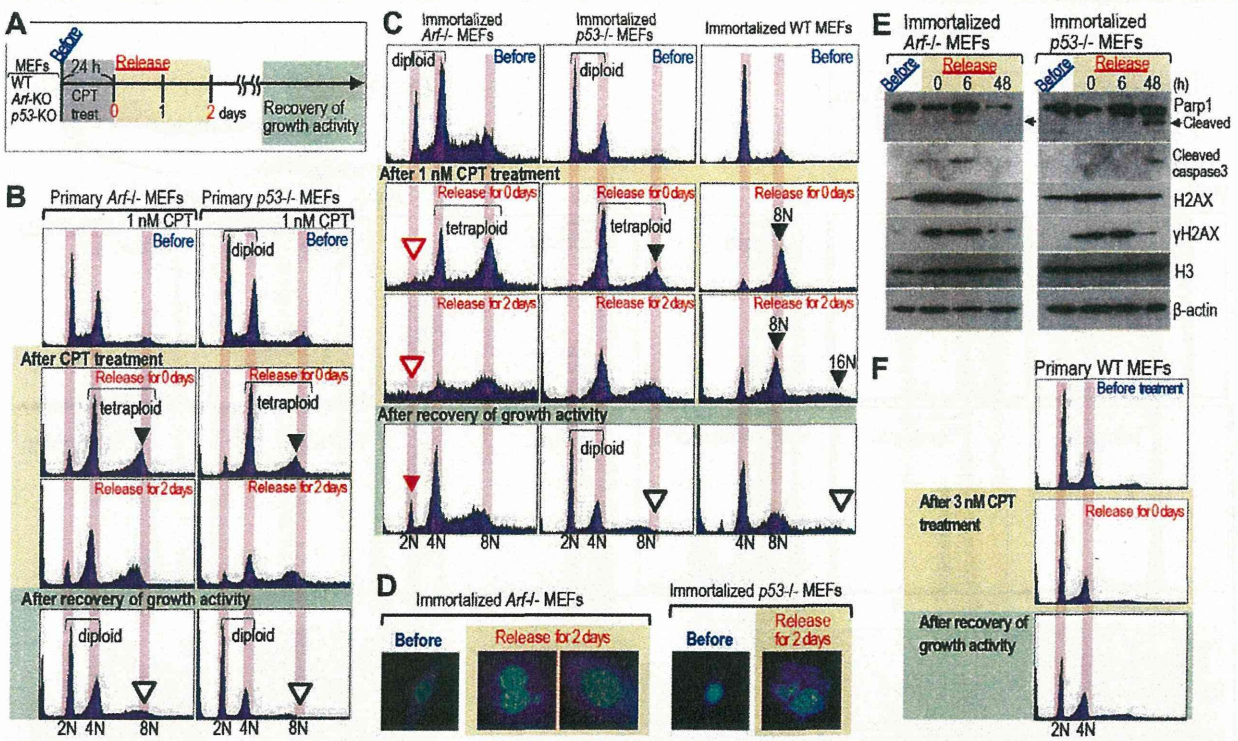

Fig. 4. Sensitivity to mitotic catastrophe affects the ploidy status of WT, $Arf^{-/-}$, and $p53^{-/-}$ MEFs. (A) WT, $Arf^{-/-}$, and $p53^{-/-}$ MEFs were exposed to CPT for 24 h and then released from treatment to induce mitotic catastrophe and to determine the effect on induction of cell death. (B) FACS analyses of primary $Arf^{-/-}$ and $p53^{-/-}$ MEFs before and after CPT treatment, and during growth recovery. These cells developed tetraploidy following exposure to CPT (solid black arrowhead) and the tetraploid cells selectively died via mitotic catastrophe during growth recovery (open black arrowheads). (C) FACS analyses of immortalized WT, $Arf^{-/-}$, and $p53^{-/-}$ MEFs before and after CPT treatment, and during growth recovery. Similar results to those seen for the primary KO MEFs were observed. In immortalized $Arf^{-/-}$ MEFs, which were a mixture of diploid and tetraploid cells (2N, 4N, and 8N chromosomes), the 2N peak was reduced following CPT treatment (open red arrowhead) and increased again upon growth recovery (solid red arrowhead). (D) Representative nuclei staining of immortalized $Arf^{-/-}$ and $p53^{-/-}$ MEFs before exposure to CPT and after release for 2 days. Multinucleated cells, which are typically formed during mitotic catastrophe cell death, were observed in $p53^{-/-}$ MEFs but not in $Arf^{-/-}$ MEFs. (E) Immunoblot analyses of the expression levels of Parp1, cleaved Parp1, caspase-3, cleaved caspase-3, H2AX, γH2AX, and histone H3 in immortalized $Arf^{-/-}$ and $p53^{-/-}$ MEFs before exposure to CPT and after release for 0 h, 6 h, or 48 h. Expression levels of β-actin were measured as a loading control. (F) FACS analysis of the ploidy status of primary WT MEFs before and after exposure to CPT. Unlike $Arf^{-/-}$ and $p53^{-/-}$ MEFs, primary WT MEFs did not undergo mitotic catastrophe or growth arrest. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tetraploidization could be induced in both *Arf*-KO and *p53*-KO MEFs (dependent on the culture conditions), this response was not associated with the development of immortality. By contrast, mutation of the *Arf/p53* module in WT MEFs is induced in association with tetraploidization [7].

3.4. Sensitivity to mitotic catastrophe determines the ploidy state of cells

In agreement with our recent study [4,7], the results described above indicate that tetraploidization is dispensable for immortalization of *Arf/p53*-mutated cells. Conversely, tetraploidization and associated mutation of the *Arf/p53* module are prerequisites for immortalization of WT MEFs, whereas cells in the diploid state are stably protected against immortalization [6]. However, the mechanisms by which tetraploidization of p53-KO MEFs is protected under growth-restricted conditions and by which tetraploidy of immortalized WT MEFs is preserved without the development of further polyploidy (such as 16N or 32N) are still unknown. The response to mitotic catastrophe, during which cells that contain DNA lesions are enter mitosis and die as they unable to maintain proper cell cycle arrest, is a mechanism that may be associated with these events [27]. Mitotic catastrophe is a major trigger of cancer cell death during treatment with anti-cancer drugs that damage DNA [28–31], whereas senescent WT MEFs with identical DNA lesions are able to progress through mitosis without undergoing cell death [4]. To investigate their sensitivity to mitotic catastrophe, MEFs were exposed to the topoisomerase I inhibitor CPT, which acts as an anti-cancer drug, and then released from treatment to induce mitotic catastrophe (Fig. 4A). Surviving cells were identified after growth-activity was recovered. FACS analysis revealed that primary *p53*-KO and *Arf*-KO MEFs underwent mitotic catastrophe with associated tetraploidization following CPT treatment (Fig. 4B, solid arrowheads). However, the 8N peak disappeared after growth recovery of these cells, indicating that the surviving cells were exclusively diploid (Fig. 4B, open arrowheads) and that selective mitotic catastrophe-associated death of the tetraploid cells had occurred.

Similar to the primary *p53*-KO and *Arf*-KO MEFs, immortalized *p53*-KO (diploid) and WT MEFs (tetraploid) underwent selective cell death via mitotic catastrophe (Fig. 4C, solid black arrowheads). For the immortalized *Arf*-KO MEFs, which were a mixture of diploid and tetraploid cells, the 2N peak disappeared after treatment with CPT (Fig. 4C, open red arrowheads) and then recovered in the surviving cells (Fig. 4C, filled red arrowhead), indicating that cells subjected to mitotic catastrophe underwent cell death. Formation of multinucleated cells, which are generally observed after mitotic catastrophe, was typically observed in immortalized *p53*-KO MEFs but not in immortalized *Arf*-KO MEFs (Fig. 4D). However, *Arf*-KO MEFs showed more efficient appearance of cleaved caspase-3 and cleaved Parp1 than *p53*-KO MEFs (Fig. 4E), suggesting efficient apoptosis induction in the *Arf*-KO MEFs. These data suggest that the mechanisms of cell death after mitotic catastrophe might be multiple and dependent on the mutation status of *Arf* and *p53*. Nevertheless, these results indicate that MEFs containing mutations of the *Arf/p53* module are highly sensitive to mitotic catastrophe, which impacts the resulting ploidy status of the cells. In fact, immortalized WT MEFs were exclusively tetraploid; therefore, mitotic catastrophe-associated cell death is probably one reason why these cells do not undergo further polyploidy (such as 16N or 32N) (Fig. 4C).

In contrast to the primary *Arf*-KO and *p53*-KO MEFs, primary WT MEFs exposed to CPT did not display signs of mitotic catastrophe, tetraploidization, or G2/M cell cycle arrest (Fig. 4F). This result suggests that the sensitivity of primary WT MEFs to mitotic catastrophe-associated cell death differs from that of the cells containing mutations in the *Arf/p53* module.

4. Discussion

This study demonstrates that both Arf and p53 are required for the establishment of an H2AX-diminished and growth-arrested state in which cells are quiescent and protected against immortalization. The results presented here indicate that mutations in either *Arf* or *p53* induce recovery of H2AX expression and development of immortality. In spite of the lack of growth arrest of *Arf*-KO and *p53*-KO MEFs, the morphology of these cells was similar to that of senescent WT MEFs. Therefore, although the Arf/p53 module is required for the establishment of a growth-arrested state and down-regulation of H2AX, proper functioning of this regulatory module is not always associated with the senescent morphological characteristics of cells.

Together with the results of our previous studies [4,7,32], the data presented here demonstrate that the cellular ploidy status is determined by at least two distinct cellular events that occur during immortalization: (i) tetraploidization-coupled induction of mutations in the Arf/p53 module, which leads to immortalization of WT MEFs and (ii) mitotic catastrophe-associated cell death, which occurs in *Arf/p53* mutated cells but is not induced in normal MEFs. Senescent WT MEFs survive after mitotic catastrophe, causing tetraploidization with mutation induction in the *Arf/p53* module, whereas cells containing mutations in the *Arf/p53* module are more sensitive to mitotic catastrophe-induced cell death. Therefore, diploid cells are protected against immortalization but tetraploid immortalized cells do not usually show further ploidy (16N or 32N).

Because tetraploidization is caused by carryover of DNA lesions through mitosis [4], immortalized tetraploid MEFs are cells that escape cell death during mitotic catastrophe. Although the mechanism that determines whether cells either escape from or undergo mitotic catastrophe-related cell death is currently unclear, tetraploid primary WT MEFs survive under accelerated growth stimuli; therefore, it is possible that impairment of the checkpoint response is involved in the process. In fact, the H2AX-diminished and growth-arrested state of primary WT MEFs is associated with an impaired checkpoint response [19]. In fact, canonical mitotic catastrophe-associated death was observed as a mechanism of cancer cell death after anti-cancer drug treatment [28–31]. Thus, primary WT MEFs that are protected from immortalization under diploidy but could escape from mitotic catastrophe with causing tetraploidization that is associated with mutation induction in Arf/p53 module. The resulting immortalized MEFs turn out sensitive to mitotic catastrophe-associated cell death due to mutation in Arf/p53 module.

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