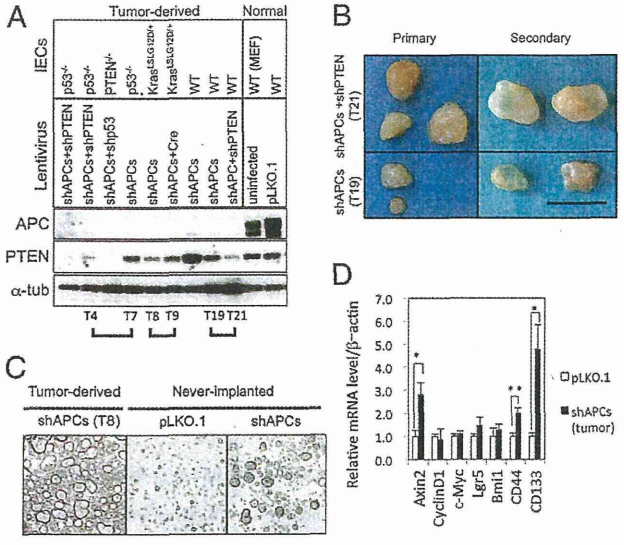

Fig. 6. Induction of cancer stem–like properties in tumors through *APC* suppression. (*A*) *APC* knockdown in tumors. Immunoblotting analysis of tumor-derived organoids for APC and PTEN. Mouse embryonic fibroblast (MEF) and normal IECs are positive controls. T4/7, T8/9, and T19/21 derived from identical primary cells, respectively. (*B*) Serial transplantation of tumors. s.c. tumors T19 (shAPCs) and T21 (shAPCs+shPTEN) in primary sites (*Left*) and corresponding secondary tumors after retransplantation (*Right*). (Scale bar, 10 mm.) (*C*) Sphere-forming assay. Representative images after 2-wk suspension culture are shown. (*D*) qPCR analysis for Wnt target genes and stem cell marker genes. Mean ± SD ($n = 3$) is shown; $*P < 0.01$; $**P < 0.05$.

engineered mouse, independently of intestinal microenvironment, and solely with primary IECs, providing an alternative way to model CRC in vitro. There were several issues to be resolved in the course of developing the model. First was to achieve stable transduction of stem cells, instead of gene targeting in embryonic stem (ES) cells. Despite initial technical difficulties, we eventually established a lentivirus-based transduction method, which was as simple as a routine subculture but highly efficient in obtaining stably transduced organoids. This methodological advantage was in sharp contrast to retroviral transduction of only cycling cells, with more complicated procedures but much less efficiency, definitely requiring drug selection to obtain stably transduced organoids (43). Both high infection efficiency and the growth advantage of inactivating tumor suppressors helped us conduct experiments without drug selection, which eliminated its potential side effects and enabled us to use vectors without selection markers or puro-resistant IECs from the *Kras*$^{LSLG12D/+}$ mouse (34). We further showed that Cre-mediated recombination of a floxed allele could be achieved in organoids. Thus, overexpression and knockdown could be simply achieved for many genes in WT IECs and gene disruption in conditionally gene-targeted IECs.

The second was in vitro expansion of *APC*-inactivated organoids. Inactivation of *APC* in the intestine is normally established by a stochastic second hit in heterozygous mice or by complete loss in mice homozygous for a floxed allele in a spatiotemporally regulated manner (22, 44, 45). Although rounded cystic organonids were available by 3D culture of *APC*-deficient adenoma developed in earlier studies (26, 27), *APC* loss and subsequent tumor development have been exclusively achieved in vivo, to which inflammation or interactions within the microenvironment might have played critical roles (17, 18). In some settings, tumor development itself could not be achieved due to organ failure induced immediately after *APC* loss (28). Thus, it was initially unclear whether we could achieve *APC* inactivation in organoids and its subsequent propagation thoroughly in vitro. IECs transduced with shAPC frequently failed to propagate, which might be partially in line with adverse effects caused by acute *APC* loss (28). We incidentally noted that this could be overcome by cointroducing all of the shAPC clones. A possible explanation could be that pooled shAPC clones yielded variations in the magnitude of Wnt activation, thereby increasing the probability of achieving the "just-right" signaling (46). Alternatively, cooperation among off-target effects by pooled clones could have contributed. Although the underlying mechanism remains to be investigated, organoids that grew out indeed carried shAPCs and phenocopied those derived from *APC*-deficient adenoma. Based on the high similarity, we reasoned that propagated organoids with shAPCs might likely be an in vitro equivalent to adenoma. We then took advantage of this situation for further analysis.

The third was the strict definition of tumor in this model, which was definitely required to relate the results to earlier studies in vivo. We tentatively defined tumors as nodules replacing coinjected Matrigel with proliferating epithelial glands at 6 wk postinjection. Only if tumors proved lethal at an earlier point were nodules exceptionally diagnosed on death. Accordingly, nonlethal nodules at 2 wk after injection were not treated as tumors. Acquisition of the potential for sphere formation and serial transplantation and induction of CSC markers were confirmed in tumor-derived organoids, clearly indicating that they had indeed undergone transformation. These results tend to support the validity of our definition of tumors.

With this experimental system, the relevance of known genetic alterations in CRC could be essentially recapitulated, either individually or in the context of *APC* loss, as in *PTEN* loss (11, 12) and *Kras* activation (13–15, 36, 37). With regard to *p53* loss, its genetic cooperation with *APC* loss was negative in the heterogeneous genetic background (31, 47) but proved to be positive in a congenic background (9, 10), consistent with this study. These findings highlight the relevance of conducting the analysis on genetic interaction in exactly the same genetic background as achieved in our model, which otherwise requires multiple backcrossing. Thus, validation of candidate genes or genetic cooperation will be warranted, leading to quick identification of the genes to be prioritized for further investigations from many candidates (19, 20) before, or even without, generation of gene-modified mice. Also, generation of tumors with defined genotypes could be facilitated. The custom-made "genetically clean" cell lines would become valuable resources for identification of effective compounds or therapeutic targets by high-throughput screening or in preclinical studies.

Considering similarities in outcome and approach, our in vitro model might well be comparable to those two types of in vivo studies, in which *APC* was subject to acute deletion in the intestine, rather than studies with the *APC*-heterozygous mutant mouse (15). One is *APC* loss in an Lgr5$^+$ stem cell–specific manner, which quickly gives rise to adenoma (22, 26, 48). The other is *APC* loss either focally or entirely in the intestine. Local injection of adenovirus-Cre induced adenoma in the distal colon, although transduction was achieved in only a limited area (44, 45). Cre-mediated inducible and acute loss of APC throughout the intestine led to morbidity within 5 d (28), but the crypts that were rescued by harvesting 2 d after APC loss proved tumorigenic in nude mice (49). In both cases, *APC* inactivation was achieved in gene-modified mice in vivo, presumably by cooperating with the microenvironment. Besides, special conditions such as cell type–specific gene ablation or crypt harvest at specific times were necessary. In contrast, in our model, *APC* inactivation was simply achieved in WT IECs in vitro, without any other type of cells or experimental conditions, obviously facilitating intestinal tumorigenesis studies. As organoid culture is optimized for Lgr5$^+$ stem cells (24), it is conceivable that Lgr5$^+$ stem cells were predominantly transformed in our model. On the other hand,

lentiviral transduction could also target quiescent stem cells, which could be marked by Bmi1 (42), Lrig1 (50), mTert (51), or HopX (52), in a mutually overlapping but distinct manner. Moreover, even Lgr5$^-$ differentiated cells could dedifferentiate to reacquire stem cell properties and initiate tumorigenesis (49). Thus, there is a possibility that tumor initiation could take place through many different pathways, including reprogramming of nonstem cells. In this regard, our model might provide unique opportunities in addressing this issue in an unbiased way, as the entire process of tumorigenesis could be simply recapitulated without predefined conditions on tumor-initiating cells.

In conclusion, we developed a unique in vitro model for CRC, with which genetic interactions in both tumor initiation and progression will be simply but genuinely analyzed. By serving as an alternative or complement to the standard approaches, it would likely accelerate CRC research.

Materials and Methods

Singly dissociated intestinal cells were lentivirally transduced in vitro. Organoids were maintained for 4 wk in Matrigel and injected into nude mice to evaluate tumorigenicity. Several weeks after the implantation, the tumors were subjected to histological analysis or 3D culture to obtain a pure population of tumor-derived organoids, which were further analyzed by Western blotting, qPCR, and sphere-forming assay. Extended materials and methods are available in *SI Materials and Methods*.

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AKT is critically involved in cooperation between obesity and the dietary carcinogen amino-1-methyl-6-phenylimidazo [4,5-*b*] (PhIP) toward colon carcinogenesis in rats

Maki Igarashi [1], Yoshitaka Hippo *, Masako Ochiai, Hirokazu Fukuda, Hitoshi Nakagama

Division of Cancer Development System, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

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ABSTRACT

Obesity is highly associated with colon cancer development. Whereas it is generally attributed to pro-tumorigenic effects of high fat diet (HFD), we here show that a common genetic basis for predisposition to obesity and colon cancer might also underlie the close association. Comparison across multiple rat strains revealed that strains prone to colon tumorigenesis initiated by a dietary carcinogen amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) tended to develop obesity. Through transcriptome and extensive immunoblotting analyses, we identified the basal level of activated AKT in colonic crypts as a biomarker for the common predisposition. Notably, PhIP induced activation of AKT, which could persist for several weeks under a low fat diet (LFD), but not under HFD. On the other hand, PhIP and HFD independently induced Wnt pathway activation and inhibited apoptosis, through distinct mechanisms involving GSK-3β, caspase 3 and poly-ADP ribose polymerase (PARP). Taken together, these observations provide mechanistic insights into how PhIP-induced activation of AKT might cooperate with HFD at multiple levels toward development of colon cancer.

1. Introduction

Colorectal cancer (CRC) is a leading cause of cancer death worldwide [1]. In the multi-step development of sporadic CRC, Wnt pathway activation is the most frequent initiating event, typically achieved by functional loss of adenomatous polyposis coli (APC) or activating mutation of CTNNB1 encoding β-catenin [2]. Subsequent progression to full-blown tumors is mediated by accumulation of genetic alterations in tumor suppressor genes and oncogenes [3], or by environmental factors, including inflammation. In fact, inflammatory bowel disease is a high-risk condition for CRC in humans [4], and dextran sodium sulfate (DSS)-induced colitis accelerates azoxymethane-induced colon tumorigenesis in mice [5]. Obesity-associated visceral fat or adipocytes have recently emerged as a source of inflammation [6]. Leptin and adiponectin, a class of cytokines secreted by adipocytes, are mediators of inflammation by binding to their specific receptors [7]. Genetic ablation of these pathways in mice indeed affected tumorigenicity under a high fat diet (HFD), confirming the pro-tumorigenic nature of obesity [8,9].

Amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) is a heterocyclic amine (HCA) abundantly contained in cooked meat. It binds to DNA and forms adducts, which could in turn induce mutations, thereby potently inducing tumors in the colon, prostate and mammary glands in rats [10]. Notably, these types of tumors are all closely associated with westernized high-fat diets in humans, and HFD indeed accelerated PhIP-initiated carcinogenesis in these organs in rats [11]. PhIP administration recapitulates multi-step colon tumorigenesis from aberrant crypt foci (ACF), dysplasia, adenoma, and adenocarcinoma [12]. Besides, PhIP-induced tumors frequently harbor mutations in APC and CTNNB1, similar to human CRC [13]. These observations strongly suggested that PhIP might be a major environmental carcinogen for human CRC.

Although ACF are not *bona fide* pre-neoplastic lesions of the colon, susceptibility of strains to chemically-induced tumorigenesis is conveniently estimated by the number of ACF at an early point, largely due to their high correlation, shorter period of time for observation, and higher incidence [14]. The numbers of ACF induced by PhIP vary among inbred strains [12], strongly suggesting that multiple genetic factors determine the susceptibility to colon carcinogenesis. In an effort to identify these loci, we noted that rat strains with more ACF tended to manifest a more severe obese phenotype, which prompted us to investigate the molecular

Abbreviations: PhIP, amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine; HCA, heterocyclic amine; ACF, aberrant crypt foci; GSEA, gene set enrichment analysis.

* Corresponding author. Fax: +81 3 3542 2530.
E-mail address: yhippo@ncc.go.jp (Y. Hippo).
[1] Present address: Department of Molecular Endocrinology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan.

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basis underlying the common predisposition. We clarified the relevance of AKT in the colonic crypts in linking obesity to PhIP-induced CRC, providing mechanistic insights into the cooperation between obesity and CRC.

2. Materials and methods

2.1. Rats, diet and chemicals

We purchased BUF, F344 and ACI rats from CLEA Japan (Tokyo, Japan), LEW, WKY and BN from Charles River Japan Inc. (Yokohama, Japan), and WKAH, OM, DA and KND from Japan SLC (Hamamatsu, Japan). PVG, DON, LEA, DRH, WF, SDJ, LE and NIG-III were provided from The National BioResource Project (NBRP) for the Rat (Kyoto University, Kyoto, Japan). Animal studies were carried out according to the Guideline for Animal Experiments, drawn up by the Committee for Ethics in Animal Experimentation of the National Cancer Center, which meet the ethical standards required by the law and the guidelines about experimental animals in Japan. Five-week-old male rats were fed a low fat diet (LFD) AIN-93G (Dyets Inc., Bethlehem, PA) for 1 week. To induce ACF, rats were fed LFD containing 400 ppm of amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) (Nard Institute, Osaka, Japan) for the first 2 weeks, followed by a high fat diet (HFD) containing hydrogenated oil PRIMEX (Dyets) for 4 weeks. To induce tumors, this cycle was repeated three times, and experimental animals were fed a HFD for the rest of the course of experiments, to conduct an intermittent PhIP feeding protocol [11]. N-acetoxy-PhIP (Nard Institute), an active form of PhIP, was used for an *in vitro* experiment.

2.2. Evaluation of obesity and tumorigenicity

After fasting for 16 h, serum and body fat were collected on sacrifice at 12 weeks of age. All the blood biochemistry data were obtained by SRL Inc. (Tokyo, Japan). Body weight and body fat weight were measured at 8, 10, and 12 weeks of age. Visceral fat was harvested from epididymal, mesenteric, perirenal and retroperitoneal fat pads. Subcutaneous fat was collected from the dorsal skin. Total body fat weight was calculated as the sum of visceral and subcutaneous fats. The colons were fixed by 10% neutralized formalin overnight and stained with 0.2% methylene blue for 15 min to count the numbers of ACF, aberrant crypts (ACs), and tumors under a stereoscope. Paraffin-embedded thin sections at 5 μm were subject to hematoxylin and eosin staining for histological analysis.

2.3. Colon crypt isolation

Colonic fragments of 1–2 cm long were washed several times with TBS, and subject to incubation at 37 °C for 30 min in Hanks' balanced salt solution supplemented with 30 mM EDTA, 5 mM PMSF, 40 mM NaF and 5 mM sodium pyrophosphate decahydrate. Isolated crypts were stored at −80 °C until used for further analysis. RNA was extracted with TRIzol reagent (Invitrogen, Tokyo, Japan). Protein was extracted with T-PER Tissue Protein Extraction Reagent (Pierce, Alabama) supplemented with Complete Mini (Roche Diagnostics, Mannheim, Germany) and Halt Phosphatase Inhibitor (Pierce).

2.4. Cell culture

Normal human colon cells FHC were cultured in media containing 10% FBS and supplemented with penicillin and streptomycin. 1 day prior to experiments, the culture supernatant was replaced with serum-free media. N-acetoxy-PhIP, an activated form of PhIP, was dissolved in DMSO and added to the cells at 10 μM.

2.5. Microarray analysis

Labeled cDNA synthesized from 500 ng of total RNA was hybridized with Agilent Whole Rat Genome 4x44K microarrays, G4131F (Agilent Technologies), following the manufacturer's instructions. Hybridization images were scanned by High Resolution Microarray Scanner (Agilent Technologies), and analyzed with Agilent Feature Extraction Software v9.5. Raw data were analyzed by Gene Spring GX 7.3.1. Gene set enrichment analysis (GSEA) was conducted with GSEA software [15].

2.6. Western blotting

The proteins were separated by SDS–PAGE and transferred to PVDF membranes. The primary antibodies against p-AKT (Ser473), AKT, FOXO1, FOXO3a, FOXO4, Bim, Caspase-3, p-GSK3β (Ser9), GSK3β, p-β-catenin (Ser33/37/The41) and non-p-β-catenin (Ser33/37/Thr41) were purchased from Cell Signaling Technology (Danvers, MA), and those against β-catenin and c-myc were purchased from BD Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology Inc., (Santa Cruz, CA), respectively. After incubation with HRP-conjugated secondary antibodies, images were visualized by enhanced chemiluminescence (Pierce). Signal intensity for p-AKT and total AKT was quantified by LAS3000 (Fujifilm, Tokyo, Japan).

2.7. Statistical analysis

All data are shown as mean ± SD. Statistical significance was determined by Mann–Whitney's *U*-test with the software JMP 9.0 (SAS Institute Japan, Tokyo, Japan). *p*-values less than 0.05 were considered significant.

3. Results

3.1. F344 rats are more susceptible to PhIP-induced colon tumorigenesis and obesity than ACI rats

Whereas carcinogenicity of chemicals is generally correlated with the number of ACF induced at an early point [16], this relationship remains elusive for PhIP. To address this issue, we chronologically monitored the colons from two rat strains treated with an intermittent PhIP-feeding protocol [11] (Fig. 1A). The numbers of both ACF and aberrant crypts (ACs) were significantly higher in the F344 rats compared to those in ACI rats at 12 weeks of age (Fig. 1B), consistent with a previous report [12]. At later time points, the number of ACs was still significantly higher in the F344 rats than in the ACI rats, but not with ACF. At 38 weeks of age, the number of colon tumors was significantly higher in F344 rats (Fig. 1C). In addition, the total number of dysplastic ACF, adenoma and adenocarcinoma were all higher in F344 rats. These results clearly indicated that the F344 rats more potently develop more advanced lesions than the ACI rats at any time point. Consequently, we reasoned that the number of ACF at 12 weeks of age would in fact serve as a marker to estimate tumor susceptibility of the strain and was used in subsequent analyses.

While examining ACF, we noted that the F344 rats tended to have more fat than the ACI rats. To verify this notion, we strictly quantified the fat weight of both strains at 6–12 weeks of age under LFD. Despite the similar level of body weight, a significantly higher degree of fat deposition was observed in F344 rats (Fig. 1D). This was also the case for visceral and subcutaneous fat. Severe accumulation of visceral fat has been associated with metabolic syndrome, which is characterized by hyperlipidemia, hypercholesterolemia and type II diabetes [17]. We then conducted

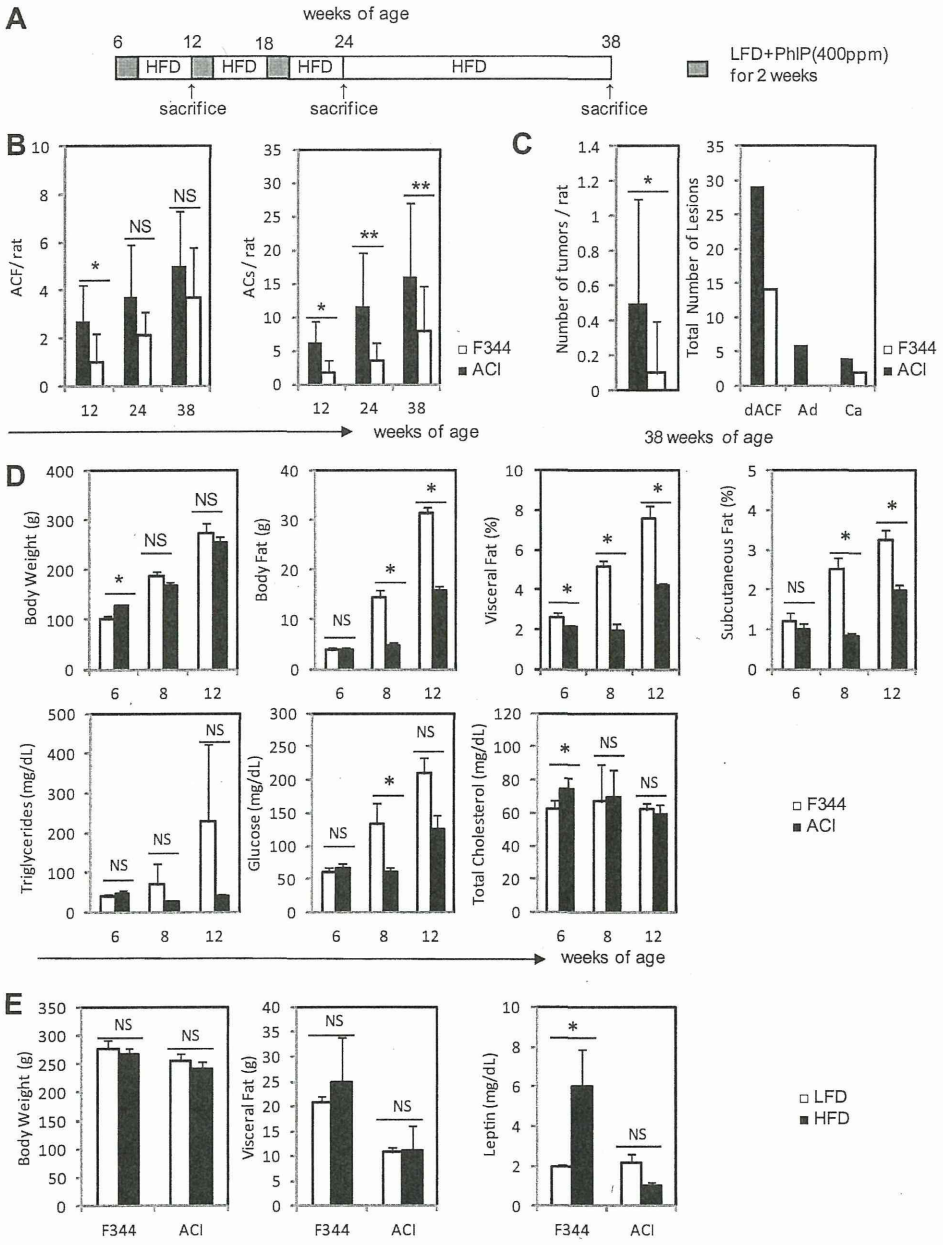

Fig. 1. Higher susceptibility to PhIP-induced colon tumorigenesis and obesity in F344 rats than in ACI rats. (A) A schematic view of the intermittent protocol for PhIP-induced colon carcinogenesis. (B) Time-series analysis of the number of ACF and ACs. The colons were examined at 12 (n = 10 each), 24 (n = 10 each), and 38 (n = 20 each) weeks of age. (C) Total number of tumors at 38 weeks of age. Both adenoma and carcinoma were counted as tumors. dACF, dysplastic ACF. Ad, adenoma. Ca, adenocarcinoma. (D) Time series analysis of body fat weight and blood biochemistry. Rats under LFD were sacrificed at 6 (n = 4 each), 8 (n = 4 each), and 12 (n = 3 each) weeks of age. (E) The effects of HFD on obesity. F344 (n = 4 each) and ACI (n = 5 each) rats under LFD or HFD for 6 weeks were sacrificed at 12 weeks of age. *p < 0.05 NS, not significant.

a blood biochemistry test and found that the level of serum triglycerides (TG) and glucose, but not total cholesterol, tended to be higher in F344 (Fig. 1D). Given that PhIP-induced colon carcinogenesis is promoted by HFD, we examined the effects of 6-week HFD on obesity. During 6–12 weeks of age, neither body weight nor the amount of visceral fat was affected in either strain (Fig. 1E). By contrast, the level of serum leptin significantly increased in F344 rats under HFD (Fig. 1E), in line with increased fat intake and ruling out the possibility that rats were improperly fed. These results indicated that F344 rats are inherently more prone to both CRC and obesity than ACI rats, which could be evaluated by measuring ACF and TG at 12 weeks of age.

3.2. Correlation between the magnitude of obesity and the incidence of ACF across multiple strains

We wondered if the observed correlation between predisposition to obesity and CRC could be more generalized. In an effort to identify genetic determinants of susceptibility to PhIP-induced CRC, we had characterized a total of 18 independent rat strains in terms of incidence of ACF under HFD for 4 weeks and collected blood samples, albeit under non-fasting conditions, from rats under LFD for 4 weeks (Fig. 2A). Although these data and samples may not be ideal for accurate analysis, we took advantage of this situation to gain insights into the common predisposition. Plotting the incidence of ACF (Fig. 2B)

and serum lipid level for each strain revealed a correlation between incidence of ACF and TG, but not cholesterol (Fig. 2C). Out of the 18 strains, we selected six strains, readily available and with relatively strong correlations, for more detailed analyses under strict conditions. Specifically, BUF, LEW, F344, and LEA, ACI, NIG-III, were postulated to constitute a tumor- and obesity-prone subgroup and a resistant subgroup, respectively. Both body fat weight and body fat percentage, with the exception of LEW rats, were indeed high in a tumor- and obesity-prone subgroup (Fig. 2D). Similar results were obtained for TG levels in a fasting state, but not cholesterol or glucose levels (Fig. 2E). These results suggested that predisposition to obesity and PhIP-induced colon tumorigenesis in the five strains might be regulated by a common mechanism.

3.3. Correlation between the magnitude of AKT activation and incidence of ACF

To gain insights into the molecular basis for the common predisposition, we set out to determine genes differentially expressed in the colons between F344 and ACI, under LFD and without PhIP. We performed Gene Set Enrichment Analysis (GSEA), which revealed a number of differentially expressed pathways (Table S1). We focused on the PI3K/Akt pathway (Fig. 3A) on the list, because it has been already implicated in both carcinogenesis and metabolism [18]. As many upstream regulators and downstream effectors of the PI3K/Akt pathway are subject to regulation by phosphorylation, we examined the level of ~30 proteins in the pathway for total protein and phosphorylated protein by Western blot analysis. We eventually found that the magnitude of AKT activation had a good correlation with colon tumor susceptibility among the five selected rat strains (Fig. 3B). In line with this observation, FOXOs and Bim, pro-apoptotic molecules inhibited by AKT, were downregulated in the crypts from the strains with higher tumor susceptibility (Fig. 3C).

3.4. Activation of AKT in the colonic cells by PhIP in vivo and in vitro

Having confirmed the static link between the common predisposition and the level of activated AKT in a basal condition, we next investigated whether PhIP and/or HFD could dynamically regulate the magnitude of AKT activation in the colon. To achieve the highest sensitivity in detecting any alterations, we selected BUF rats, which manifested the most pronounced AKT activation (Fig. 3B). Western blotting revealed that AKT was hyper-activated exclusively in colonic crypts from the subgroup treated by PhIP for 2 weeks, followed by LFD for 4 weeks (Fig. 4A). In line with this

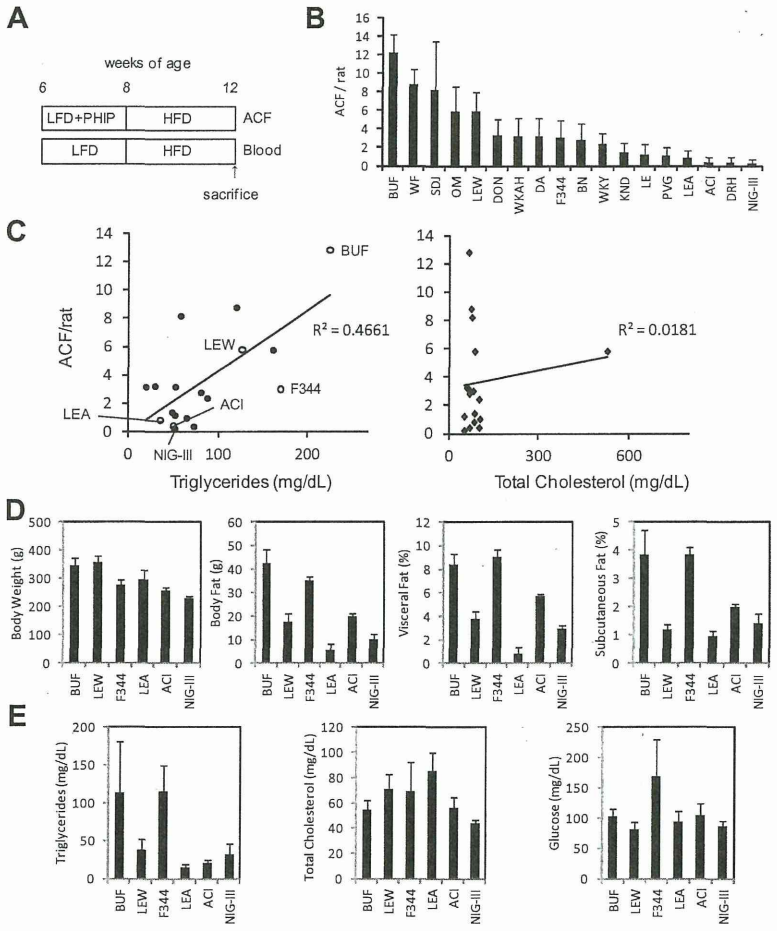

Fig. 2. Correlation between susceptibility to obesity and PhIP-induced colon tumorigenicity. (A) Feeding protocols. (B) The number of PhIP-induced ACF across 18 rat strains ($n = 5$ or 6 each). (C) Correlation between the number of ACF and amount of serum lipid. Triglyceride (*left*), and total cholesterol (*right*). Each circle depicts the mean level of serum lipid taken in a non-fasting condition from 5 individuals of each strain. Open circles labeled by strain name were used in the subsequent analysis D and E. Evaluation of obesity (D) and blood biochemistry (E) in six selected strains. Rats under LFD without PhIP for 2 weeks and subsequently under 4 weeks of HFD were examined ($n = 5$–11 each). BUF and F344, but not LEW, manifested an obesity phenotype, while ACI, LEA, and NIG-III did not (D). Only the level of serum triglyceride exhibited higher in BUF and F344, compared to the others (E).

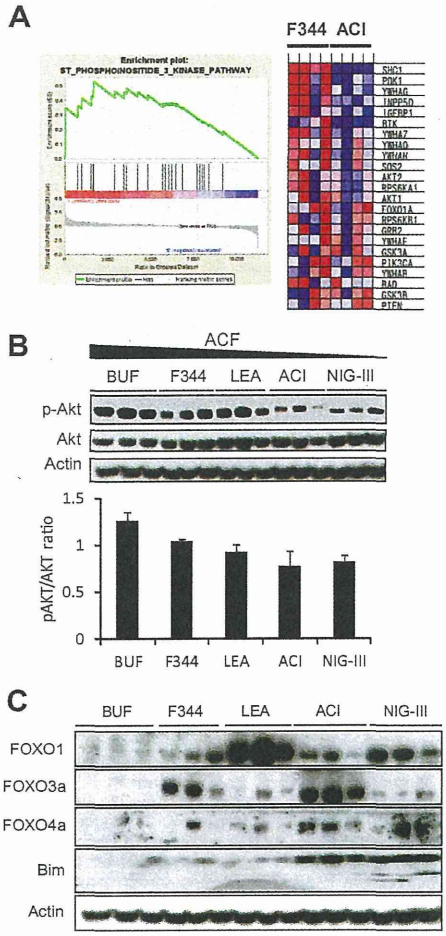

Fig. 3. Basal levels of pAKT in the colon correlate with predisposition to obesity and colon cancer. (A) Gene set enrichment analysis. The colonic crypts from F344 and ACI rats ($n = 4$ each) were subject to microarray analysis. Genes in the PI3K pathway overrepresented in differentially expressed genes (*left*). A heat map showing PI3K pathway genes (*right*). (B) Correlation between magnitude of AKT activation and the incidence of ACF. Western blotting analysis ($n = 3$) of the colonic crypts for total AKT, p-AKT (*upper panel*). Signal intensity ratio of p-AKT to total AKT correlated with the number of PhIP-induced ACF (*lower panel*). (C) Expression of pro-apoptotic molecules downstream of AKT. Western blotting analysis ($n = 3$) revealed FOXO family genes and Bim tended to show lower expression in tumor-prone rat strains. β-actin serves as a loading control.

observation, GSK-3βa key substrate of AKT, was phosphorylated specifically in the same group. The increase of pAKT was marginal without PhIP treatment or under HFD, even after PhIP treatment. To determine if the activation is achieved by a direct effect of PhIP on colonic cells, we treated human normal colon cells FHC with 10 μM of acetoxy-PhIP, a biologically active form of PhIP. Phosphorylation of AKT was indeed observed *in vitro*, albeit at a very early point and in a transient manner (Fig. 4B). These results implied that PhIP directly and promptly activates AKT, which could be sustained *in vivo* only under LFD, by an unknown mechanism.

3.5. PhIP and HFD inhibited apoptosis and activated Wnt pathway by distinct mechanisms

Given that GSK-3β promotes degradation of β-catenin, inactivation of GSK-3β by AKT is supposed to result in β-catenin accumulation leading to Wnt pathway activation. Indeed, PhIP-induced AKT activation increased the amount of total β-catenin, consistent with an earlier study [19], but to a lesser extent compared to HFD

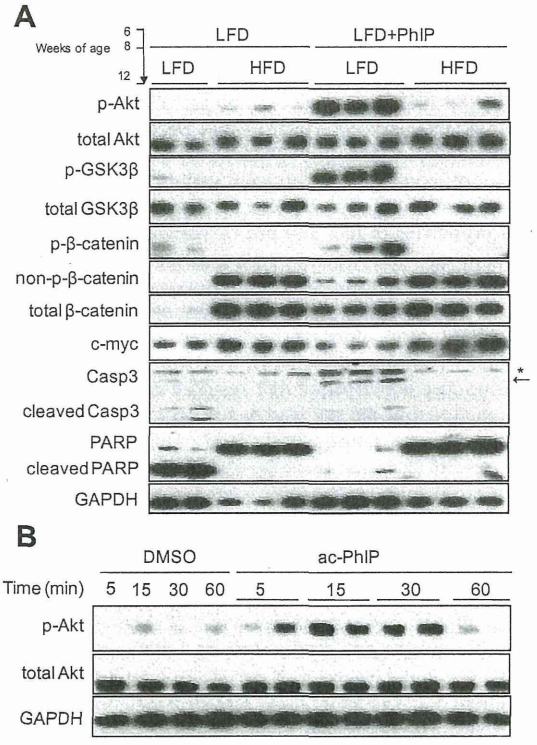

Fig. 4. Wnt pathway activation and inhibition of apoptosis by PhIP and HFD. (A) Characterization of key molecules in the Wnt pathway and apoptosis. Colonic crypts of the BUF rats from 4 subgroups ($n = 3$ each, $n = 2$ for LFD with PhIP) were analyzed by Western blotting analysis. GAPDH serves as a loading control. Non-specific bands (*asterisk*), specific bands (*arrow*) for full-length caspase3. Note that effects by PhIP were sustained even at 4 weeks later under LFD, but not under HFD. (B) Activation of AKT by PhIP in vitro. Normal human colon cells FHC were exposed to 10 μM N-acetoxy-PhIP (ac-PhIP). Negative controls were treated with DMSO ($n = 1$). Cells were collected at 5, 15, 30, and 60 min after PhIP treatment ($n = 2$).

(Fig. 4A). To qualitatively characterize β-catenin, we examined its phosphorylated and non-phosphorylated form, corresponding to an inactive and active form, respectively. It was revealed that HFD exclusively increased the amount of active β-catenin, while PhIP predominantly increased the amount of inactive β-catenin. In line with this observation, the level of c-myc, a major Wnt target gene, was indeed higher under HFD than upon PhIP treatment (Fig. 4A). AKT-induced elevation of inactive β-catenin, however, contradicts with the assumption that inactivation of GSK-3β should result in accumulation of active β-catenin, strongly suggesting that an alternative mechanism might be operating.

We next examined the effects of PhIP and HFD on apoptosis. Caspase3 and poly-ADP ribose polymerase (PARP) were predominantly in cleaved forms in colonic crypts under LFD, indicating massive apoptosis. By contrast, the cleaved forms were not detected under HFD or treated by PhIP, which seems to be achieved via distinct mechanisms. HFD suppressed expression of caspase3, thereby diminishing its cleaved form, while PhIP suppressed cleavage from full-length caspase3. Conversely, PhIP regulated PARP and caspase3 in a reciprocal manner (Fig. 4A). Collectively, HFD and PhIP activated the Wnt pathway and inhibited apoptosis, but through distinct mechanisms in the colon.

4. Discussion

Obesity, a major risk for CRC, has been generally implicated in progression from the initiation step of carcinogenesis. In the

present study, we showed that obesity could be also implicated in the early stages, by sharing a common genetic predisposition with PhIP-induced tumorigenesis. The common genetic predisposition appeared to be conveniently estimated by the level of serum TG and activated AKT in the colonic mucosa. AKT was also dynamically activated by PhIP, which seemed to be promoted by the intestinal microenvironment, but inhibited by HFD, underscoring the relevance of cooperation between genetic and environmental factors toward PhIP-induced colon carcinogenesis. Given the pro-survival properties of AKT and pro-tumorigenic effects of obesity, inhibition of AKT activation by HFD appears paradoxical in terms of tumor promotion. However, this observation might account for the reason why a cycling protocol alternating PhIP with HFD could induce colon tumors more efficiently than continuous exposure to PhIP [11].

PhIP-induced activation of AKT resulted in inactivation of GSK-3β as predicted, but did not lead to full activation of β-catenin for an unknown reason. Consequently, PhIP + LFD induced only a modest increase of the non-phosphorylated β-catenin compared to HFD. These results imply Wnt pathway-independent roles of GSK-3β inhibition in PhIP-induced tumorigenesis. In support of this notion, the colony formation potential of singly dissociated intestinal stem cells in 3D culture is significantly improved by a GSK-3β inhibitor, but not by Wnt3a ligands [20], raising the possibility that PhIP could promote survival of stem cells that might harbor mutations introduced by PhIP. Both PhIP and HFD inhibited apoptosis of colonic crypts, but surprisingly in a completely distinct manner that has never been reported previously. Expression of PARP was suppressed by PhIP, but induced and retained by HFD. As PARP is a component of the TCF4/β-catenin complex and positively regulates its transcriptional activity [21,22], its presence might contribute to a more pronounced activation of the Wnt pathway by HFD than by PhIP.

Considering high serum TG has recently emerged as a high risk factor for CRC in humans [23] [24], consistent with the present study, the findings from this study might have implications on personalized medicine. For instance, those individuals with high serum TG and AKT phosphorylation in the colon might constitute a subgroup with higher risk for CRC, even in the absence of macroscopic colonic lesions. Development of biomarkers for downstream of AKT would be also warranted, which would enable efficient reduction of cancer risk by patient education, early detection of cancer and therapeutic intervention. Taken together, we demonstrated the relevance of AKT in the development of PhIP-induced and obesity-related CRC, providing not only mechanistic insights, but also clinical implications on the diagnosis and prevention of CRC.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.059.

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Review Article

RNAi Therapeutics and Applications of MicroRNAs in Cancer Treatment

Keita Uchino[1,2], Takahiro Ochiya[1] and Fumitaka Takeshita[1,*]

[1]Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Tokyo and [2]Department of Biological Information, Tokyo Institute of Technology, Yokohama, Kanagawa, Japan

*For reprints and all correspondence: Fumitaka Takeshita, Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan. E-mail: futakesh@ncc.go.jp

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RNA interference-based therapies are proving to be powerful tools for combating various diseases, including cancer. Scientists are researching the development of safe and efficient systems for the delivery of small RNA molecules, which are extremely fragile in serum, to target organs and cells in the human body. A dozen pre-clinical and clinical trials have been under way over the past few years involving biodegradable nanoparticles, lipids, chemical modification and conjugation. On the other hand, microRNAs, which control the balance of cellular biological processes, have been studied as attractive therapeutic targets in cancer treatment. In this review, we provide an overview of RNA interference-based therapeutics in clinical trials and discuss the latest technology for the systemic delivery of nucleic acid drugs. Furthermore, we focus on dysregulated microRNAs in human cancer, which have progressed in pre-clinical trials as therapeutic targets, and describe a wide range of strategies to control the expression levels of endogenous microRNAs. Further development of RNA interference technologies and progression of clinical trials will contribute to the achievement of practical applications of nucleic acid drugs.

Key words: RNA interference – microRNA – DDS – Cancer

INTRODUCTION

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants initiated by double-stranded RNA (dsRNA). It is the most significant recent contribution to the field of cell biology, and Fire and Mello who discovered it were awarded the Nobel Prize for Medicine in 2006 (1). The silencing technology to suppress the expression of pathologically or physiologically important genes by using small interfering RNA (siRNA) is applicable to many kinds of research or therapeutics for human diseases caused by specific genes, which are difficult to regulate through traditional approaches. Indeed, as the initial description of RNAi in animals, the development of RNAi-based therapies has provided a powerful new arsenal against various human diseases, such as age-related macular degeneration (AMD) (2,3), respiratory syncytial virus (RSV) infection (4), neurodegenerative disorders (5) and cancers (6–8).

On the other hand, in recent years, microRNAs (miRNAs) have been studied as regulators of gene expression in crucial biological processes, including cell development, differentiation, apoptosis and proliferation (9,10). miRNAs are non-coding small RNAs (~22 nt) which are processed from endogenously expressed transcripts and induce translational suppression and mRNA degradation in animals, plants and viruses (11,12). miRNAs are first transcribed as primary miRNA (pri-miRNA) transcripts by RNA polymerase II and then processed by Drosha in the nucleus to generate

~60–100 nt precursor-miRNA (pre-miRNA) with a hairpin-like structure. After pre-miRNAs are transported to the cytoplasm by Exportin-5, they are processed into mature miRNA duplexes by Dicer assembled with transactivating response RNA-binding protein and protein activator of PKR (13,14). Finally, one strand of the mature miRNA duplex, a guide strand, is incorporated into the Argonaut-containing RNA-induced silencing complex, which induces either cleavage or translational repression of targeted mRNAs based on their sequences (Fig. 1). Once the miRNAs are unbalanced or the functions are disordered, they can be involved in the initiation and development of fatal human ailments, including cancer (15). Indeed, many reports have shown that the widespread disruption of miRNAs was correlated with the initiation and progression of human cancer and demonstrated that an injection with synthetic RNA molecules mimics tumor suppressor miRNAs or the inhibitors of oncogenic miRNA (onco-miR) can switch dozens of cancer-related signals on or off (16). In other words, miRNAs are potential therapeutic tools for cancer treatment, representing a superior molecular target approach to the traditional low-molecular compound approach. However, for the realization of RNAi-based therapies using siRNAs, synthetic miRNAs and miRNA inhibitors, more continuous improvements will be required. For example, the technology to avoid unwanted innate immune responses, instability of nucleic acid *in vivo* and off-target side effects strikingly decreases the levels of potency and efficacy of RNAi effector molecules (17–19). Thus, the development of drug delivery systems (DDS) for RNAi therapeutic strategies that are safer, more stable and more effective is a paramount consideration.

Although clinical applications of RNAi-based therapies have not been fully realized, numerous pre-clinical studies in animal models of human disease are providing opportunities for practical use. In this review, we provide an overview of the current clinical and pre-clinical trials of RNAi therapies and discuss strategies toward a pathway of miRNA to practical applications for cancer therapy from the viewpoint of RNAi DDS.

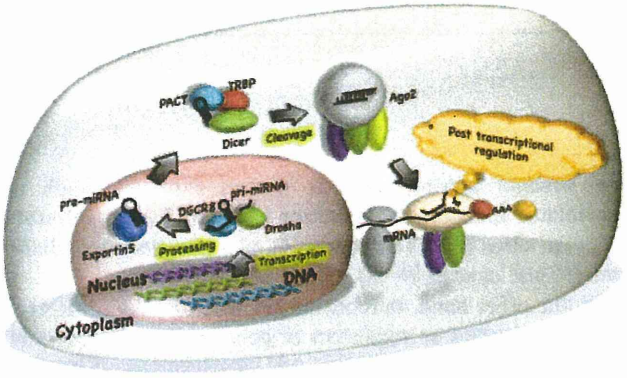

Figure 1. Cellular mechanisms of RNA interference pathway in mammals. First, primary miRNAs (pri-miRNAs) are transcribed by RNA polymerase II and are cleaved by the enzyme Drosha into ~70 nucleotides as precursor-miRNAs (pre-miRNAs). Next, these pre-miRNAs are exported to the cytoplasm with Exportin-5 and are cleaved to double-strand RNAs that do not contain a loop by Dicer. These duplexes are then associated with Argonote2 (Ago2), and one strand is removed. This RNAi-induced silencing complex (RISC) containing the guide strand triggers post-transcriptional regulation of target mRNA depending on the seed sequence of miRNAs.

RNAi THERAPEUTICS DEVELOPMENT PIPELINE

In the development of RNAi technology for therapeutic medication, since the first demonstration of RNAi triggered by siRNA in mammalian cells in 2001 (20,21), some risk-taking biotechnology companies, such as Sirna Therapeutics, Silence Therapeutics and Tekmira, started to establish a platform to develop the new technology using primarily siRNA. At first, some pharmaceutical companies ascribed the RNAi to research for directed gene silencing; however, after the first exploration of *in vivo* gene knockdown (22), major pharmaceutical firms, such as Medtronic, Novartis and Merck, became involved in clinical applications. Observers were surprised by the acquisition by Merck and Roche of Sirna Therapeutics for more than one billion USD. RNAi was considered an exceptional technology for the knockdown of therapeutic target genes, and scientists anticipated that it would significantly shorten the drug development timeline. However, as a consequence of the global economic turmoil that began in 2008 and the slump in development of DDS for RNAi medicine, companies such as Merck, Pfizer, Abbott Labs and Roche were forced to curtail research in these fields. In particular, the fact that Roche halted its development of RNAi technology in 2010 was a shock throughout the industry. The Roche decision resulted in a loss of confidence in the company's ability to innovate, and their withdrawal from RNAi research was followed by other companies. However, the clinical pipeline of RNAi therapies using siRNA has been gradually growing since approximately 2011 as the RNAi technology has matured.

As shown in Table 1, there are many candidates for clinical development in 2012. In particular, there are a number of sites for topical or local administration, such as the skin, retina and airways, which permit safe and efficient delivery without unwanted side effects. For example, according to some recent animal experiments, transtympanic administration of siRNA targeting NOX3 is significantly useful for the attenuation of cisplatin ototoxicity (23). Furthermore, Paller *et al.* (24) at Northwestern University showed that spherical nucleic acid nanoparticle conjugates gold cores surrounded by immobilized siRNA directed against EGFR can be topically delivered more stably into mouse and human skin without undesirable toxicity. Thus, accessibility is a key requirement for successful RNAi *in vivo* to be delivered tissue or cell specifically. Since around 2008, however, the development pipeline has shifted from local to systemic delivery because more advanced delivery vehicles for systemic