Table I. Final incidence and multiplicity data for mammary tumors.

	+/+ genotype				+/*fa* genotype			
	Basal diet (n=16)		Corn oil diet (n=16)		Basal diet (n=33)		Corn oil diet (33)	
	Incidence (%)	Multiplicity (No./rat)	Incidence (%)	Multiplicity (No./rat)	Incidence (%)	Multiplicity (No./rat)	Incidence (%)	Multiplicity (No./rat)
Carcinoma	7 (44)	0.88±1.41[a]	8 (50)	0.69±0.79	20 (61)	1.30±1.49	19 (58)	0.94±1.27
Adenoma	1 (6)	0.06±0.25	1 (6)	0.06±0.25	0	-	1 (3)	0.03±0.17
Fibroadenoma	4 (25)	0.25±0.45	3 (19)	0.25±0.58	7 (21)	0.30±0.64	5 (15)	0.24±0.61
Fibroma	0	-	0	-	2 (6)	0.06±0.24	0	-

[a]Means ± SDs.

Table II. Final volumes of mammary tumors.

	+/+ genotype				+/*fa* genotype			
	Basal diet (n=16)		Corn oil diet (n=16)		Basal diet (n=33)		Corn oil diet (n=33)	
	No. of tumors	Volume (cm^3/tumor)	No. of tumors	Volume (cm^3/tumor)	No. of tumors	Volume (cm^3/tumor)	No. of tumors	Volume (cm^3/tumor)
Carcinoma	14	2.06±4.14	11	6.72±8.89	43	2.92±6.29	31	6.86±12.14
Adenoma	1	0.01	1	0.21	0	-	1	0.11
Fibroadenoma	4	0.26±0.23	4	18.80±31.95	10	1.49±2.54	8	4.97±13.34
Fibroma	0	-	0	-	2	35.56±50.21	0	-

Values are means ± SDs.

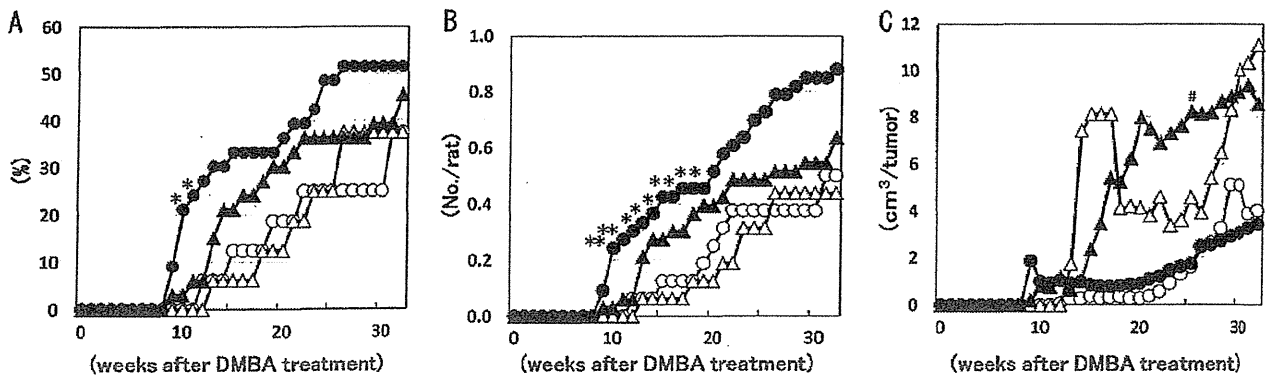

Figure 2. Sequential changes in palpable mammary carcinomas. (A) Cumulative incidence of rats with carcinomas; (B) cumulative mean number of carcinomas per rat (multiplicity); (C) cumulative mean volume of nodule/mass of carcinomas. Open circles, +/+-basal diet; open triangles, +/+-10% corn oil diet; closed circles, +/*fa*-basal diet; closed triangles, +/*fa*-10% corn oil diet. *P<0.05, **P<0.01 vs. +/+-basal diet (difference with the genotype basis); #P<0.05 vs. +/*fa*-basal diet (difference with the diet basis).

Final incidence, multiplicity and volume of mammary tumors. Incidence, multiplicity and volume findings for histopathologically defined mammary tumors are summarized in Table I. Histopathologically, mammary tumors could be classified as adenocarcinomas and benign lesions, such as adenomas, fibroadenomas and fibromas. Incidence and multiplicity of mammary carcinomas showed a tendency for increase (~1.5-fold) in the +/*fa*-basal diet group as compared with +/+-controls, but no influence on the genotype was noted in the corn oil diet groups. Furthermore, the corn oil diet showed no apparent effect on the incidence and multiplicity of mammary carcinomas in each genotype. Incidence and multi-

Table III. Distribution of sub-classified mammary carcinomas based on the morphological phenotypes among the groups.

	+/+ genotype				+/*fa* genotype			
	Basal diet (n=16)		Corn oil diet (n=16)		Basal diet (n=33)		Corn oil diet (n=33)	
	Incidence (%)	Multiplicity (No./rat)	Incidence (%)	Multiplicity (No./rat)	Incidence (%)	Multiplicity (No./rat)	Incidence (%)	Multiplicity (No./rat)
Moderately/poorly differentiated carcinoma with atypia	1 (6)	0.06±0.25[a]	0	-	6 (18)	0.21±0.48	3 (9)	0.09±0.29
Well-differentiated carcinoma without distinct atypia	7 (44)	0.81±1.22	8 (50)	0.69±0.79	16 (48)	1.09±1.49	17 (52)	0.85±1.28

[a]Means ± SDs.

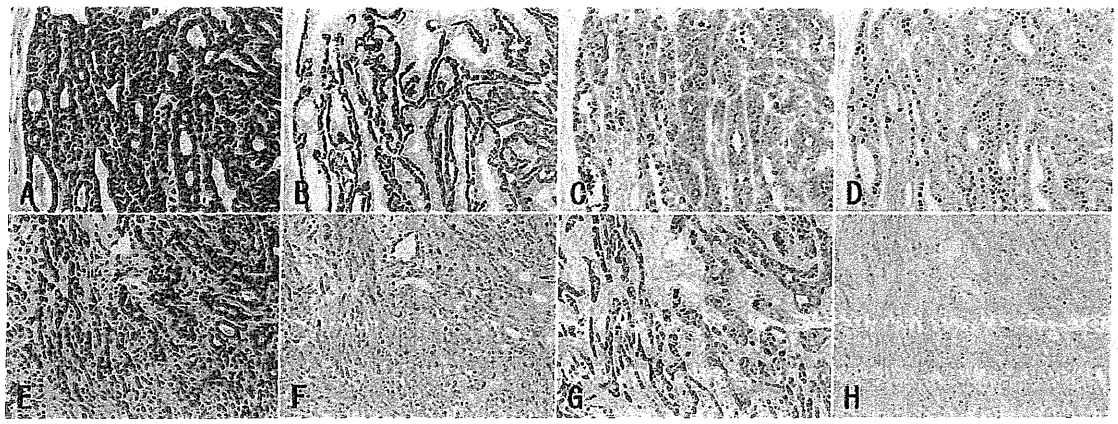

Figure 3. Histopathology and immunohistochemistry of mammary carcinomas. (A-D) A well differentiated carcinoma without distinct atypia in a +/+ rat fed basal diet; (E-H) a moderately/poorly differentiated carcinoma with atypia in a +/*fa* rat fed basal diet. (A and E) H&E. Immunohistochemistry for (B and F) α smooth muscle actin, showing cytoplasmic positivity for myoepithelial cells; (C and G) leptin receptor membranous positivity in carcinoma cells; (D and H) estrogen receptor α nuclear positivity in carcinoma cells. Original magnification, x360.

plicity of adenomas, fibroadenomas and fibromas were similar among the groups. On the other hand, volume of mammary carcinomas as well as fibroadenomas showed a tendency for increase by the corn oil diet with both +/+ and +/*fa* genotypes (Table II).

Histopathology, immunohistochemistry and western blot analysis of mammary carcinomas. Mammary adenocarcinomas found in the present experiment were mainly well differentiated without distinct nuclear atypia; however, some carcinomas showed moderately/poorly differentiated phenotypes with nuclear atypia (Fig. 3A and E). Well differentiated carcinomas showed papillotubular structures with cribriform patterns, and the tubules were generally well demarcated with α smooth muscle actin-positive myoepithelial cells (Fig. 3B). On the other hand, moderately/poorly differentiated carcinomas showed distinct invasion with small cord/glandular or scattering patterns mainly in the peripheral portion, and interstitial cell proliferation was prominent (Fig. 3F). The distribution of the sub-classified mammary carcinomas based on the morphological phenotypes among the groups is summarized in Table III. Incidence and multiplicity of moderately/poorly differentiated carcinomas with atypia showed a tendency for increase in the +/*fa*-basal diet and+/*fa*-corn oil diet groups as compared with their +/+-counterparts (Table III). The latency period of moderately/poorly differentiated carcinomas with atypia was shorter than that of well differentiated carcinomas without distinct atypia in the +/*fa*-basal diet group (Fig. 4).

To clarify expression profiles of leptin- and estrogen-related proteins in the well differentiated carcinomas without distinct atypia and moderately/poorly differentiated carcinomas with atypia, immunohistochemical and immunoblot analyses were performed. Mammary carcinomas of both phenotypes showed various expression intensities for leptin receptors (Fig. 3C and G) and leptin (data not shown), whereas the cases with atypia showed lower ER α-positivities than those without distinct atypia in the +/*fa*-basal diet and +/*fa*-corn oil diet groups (Table IV, Fig. 3D and H). For ER

Table IV. Estrogen receptor (ER) α-positivity in sub-classified mammary carcinomas based on the morphological phenotypes.

	+/+ genotype				+/*fa* genotype			
	Basal diet		Corn oil diet		Basal diet		Corn oil diet	
	No. of carcinomas examined	ER α-positivity (%)	No. of carcinomas examined	ER α-positivity (%)	No. of carcinomas examined	ER α-positivity (%)	No. of carcinomas examined	ER α-positivity (%)
Moderately/poorly differentiated carcinoma with atypia	1	1.0	0	-	7	8.9±3.9[b]	3	14.1±11.2
Well-differentiated carcinoma without distinct atypia	5	28.2±16.6[a]	6	24.2±14.2	6	36.4±15.0	10	28.9±11.4

[a]Means ± SDs; [b]P<0.01 vs. well-differentiated carcinoma without distinct atypia.

Table V. Serum biochemistry data at terminal sacrifice.

	+/+ genotype				+/*fa* genotype			
	Basal diet		Corn oil diet		Basal diet		Corn oil diet	
	No. of samples	Serum levels	No. of samples	Serum levels	No. of samples	Serum levels	No. of samples	Serum levels
Triglycerides (mg/dl)	16	340.8±138.7	15	311.5±221.5	31	392.3±312.6	31	279.6±146.5
Total cholesterol (mg/dl)	16	119.7±22.9	15	108.3±25.4	31	125.0±38.5	31	105.7±17.5[b]
Glucose (mg/dl)	16	134.6±15.9	15	145.3±15.4	31	133.7±13.3	31	141.9±17.5[b]
Leptin (pg/ml)	8	314.4±96.4	7	691.9±540.1	13	191.6±123.1	12	506.4±439.3[b]
Adiponectin (μg/ml)	8	6.7±1.4	7	7.1±3.8	12	4.0±1.0[a]	12	6.5±1.4[c]
Insulin (ng/ml)	8	2.1±0.8	7	1.5±1.0	14	1.3±0.9	13	1.5±1.1
IGF-I (ng/ml)	8	612.7±85.7	7	476.4±79.3[a]	12	609.6±178.4	12	535.3±96.6

Values are means ± SDs. [a]P<0.01 vs. +/+-basal diet group. [b]P<0.05, [c]P<0.01 vs. +/*fa*-basal diet group.

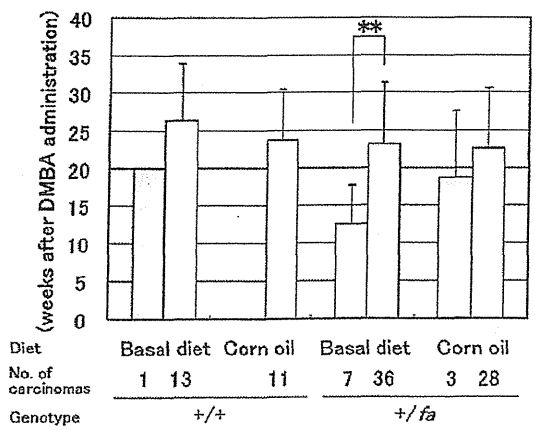

Figure 4. Latency of sub-classified mammary carcinomas based on the morphological phenotypes. Gray bars, moderately/poorly differentiated carcinoma with atypia; open bars, well-differentiated carcinoma without distinct atypia. **P<0.05.

β- and aromatase-immunohistochemistry, frozen sections of 1, 0, 3 and 3 moderately/poorly differentiated carcinomas from the +/+-basal diet, +/+-corn oil diet, +/*fa*-basal diet and +/fa-corn oil diet groups, respectively, and 2, 4, 1 and 2 well differentiated carcinomas each were used (Fig. 5A and B). Although no apparent differences in the positive intensities or positive cell ratio for ER β and aromatase were found among the combinations with two phenotypes and two diets in the immunohistochemistry, immunoblot analyses revealed a decrease in ER β expression levels in moderately/poorly differentiated carcinomas (Fig. 5C and D) and decreased expression levels of aromatase in mammary carcinomas regardless of their phenotypes and diets as compared to the normal mammary tissue (Fig. 5C and E).

To examine the relation of intracellular signaling cascades responsive to extracellular stimuli, such as growth factors or cytokines, with the mammary carcinoma phenotypes, immunoblot analyses for phosphorylation levels of ERK1/2

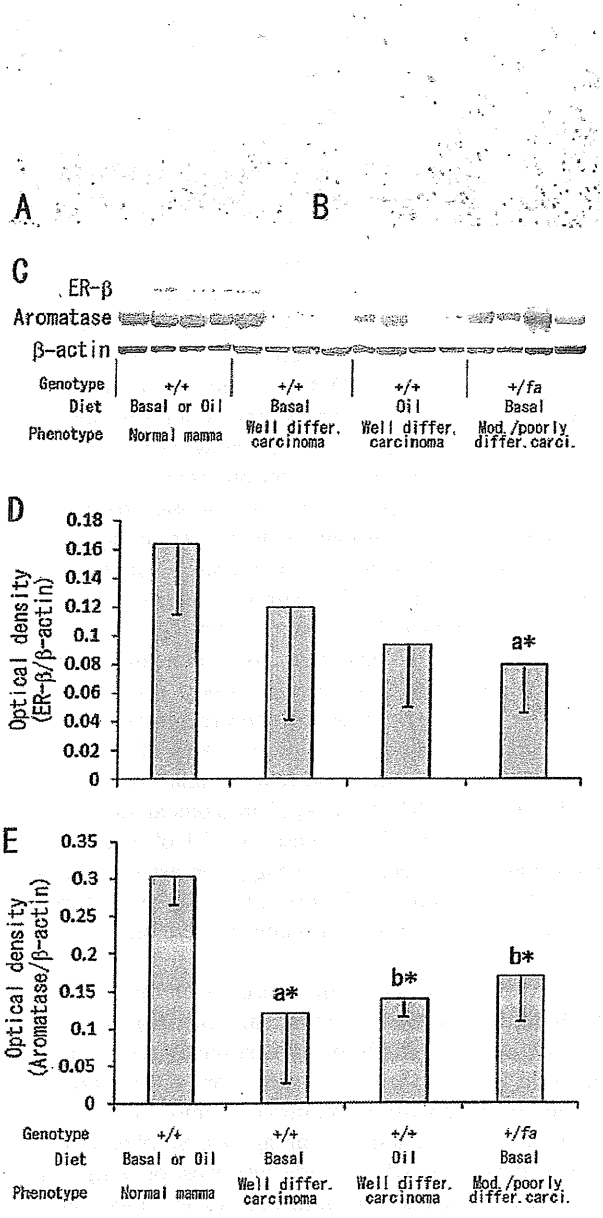

Figure 5. Expression of estrogen receptor (ER) β and aromatase in mammary carcinomas. Immunohistochemistry for (A) ER β, showing nuclear positivity in carcinoma cells of a well differentiated carcinoma in a +/+ rat fed basal diet; (B) aromatase, showing cytoplasmic positivity in presumed myoepithelial and/or mesenchymal cells of a moderately/poorly differentiated carcinoma in a +/*fa* rat fed basal diet. Western blotting for ER β and aromatase (C), and semi-quantitative optical density of ER-β (D) and aromatase (E). ^{a*}P<0.05, ^{b*}P<0.01 vs. normal mammary tissue.

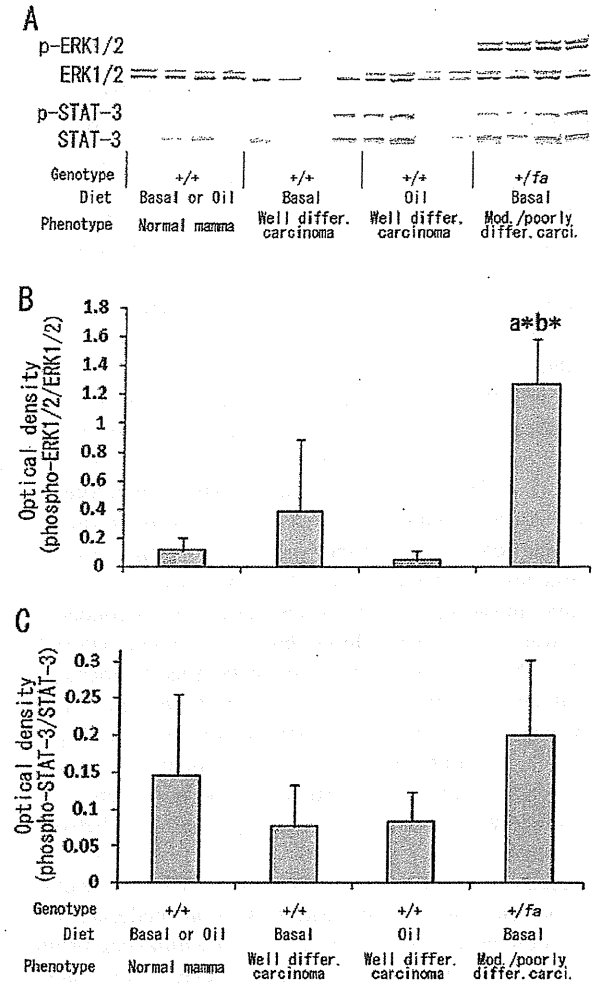

Figure 6. Phosphorylation levels of extracellular signal-regulated kinase (ERK)1/2 and signal transducer and activator of transcription (STAT)3 in mammary carcinomas. Western blotting for phospho-ERK1/2, ERK1/2, phospho-STAT3 and STAT3 (A) and semi-quantitative optical density of phospho-ERK1/2 (B) and phospho-STAT3 (C) compared to those of ERK1/2 and STAT3, respectively. ^{a*}P<0.05 vs. well differentiated carcinomas in +/+ rats fed basal diet, and ^{b*}P<0.01 vs. normal mammary tissue in +/+ rats fed basal or corn oil diet and well-differentiated carcinomas in +/+ rats fed corn oil diet.

and STAT3 were performed, and activation of the ERK1/2 signaling pathway but not STAT3 was demonstrated in moderately/poorly differentiated carcinomas with atypia as compared to normal mammary tissue and well differentiated carcinomas without distinct atypia (Fig. 6). No influence of corn oil diet was found with regard to either ERK1/2 or STAT3 activation (Fig. 6).

Serum biochemistry. Data for serum levels of triglycerides, total cholesterol and glucose at terminal sacrifice are summarized in Table V. Although triglyceride and total cholesterol levels declined or showed a tendency for decline and glucose levels were elevated by the corn oil diet in the +/*fa* genotype, no apparent change in these three parameters was observed in +/+ controls. No obvious differences in these parameters were found between the genotypes. Serum leptin levels in the +/*fa*-basal diet and the +/*fa*-corn oil diet groups were comparable to those in the +/+-counterparts, whereas corn oil diet elevated serum leptin levels in the +/*fa* genotype with a similar tendency for elevation in the +/+ genotype (Table V). Serum adiponectin levels in the +/*fa*-basal diet group were lower than in the +/+-basal diet group, and corn oil diet caused elevation only in the +/*fa* case. Serum IGF-I levels were lower in the +/+-corn oil diet than +/+-basal diet groups, but no change was observed with the +/*fa* genotype. There was no evident variation noted in serum insulin levels among the groups.

Discussion

The present DMBA-induced mammary carcinogenesis study using heterozygous (+/*fa*) and wild-type (+/+) lean Zucker rats revealed higher susceptibility of +/*fa* rats to DMBA induction of mammary tumors than +/+ rats, and also differences in histopathological phenotypes of the induced carcinomas. In particular, the latency periods of mammary carcinoma development in +/*fa* rats fed basal or corn oil diet appeared shorter than those in +/+ rats and the incidences and multiplicities of mammary carcinomas were increased or showed a tendency for increase in the early stages, with a greater percentage of more advanced cancer at the termination.

Although the body weight of +/+ and +/*fa* rats fed corn oil diet were higher than those of the rats fed basal diet, the body weight differences between +/+ and +/*fa* rats fed basal diet or corn oil diet were significantly smaller. Therefore, the short latency periods and the higher incidence and multiplicity of mammary carcinomas in the early stages in +/*fa* rats were considered not to be directly due to body weight change. On the other hand, in our preliminary study, serum leptin concentration at 7 weeks of age was ~140 pg/ml in +/*fa*, higher ($P<0.01$) than ~80 pg/ml (18). These results indicated that the increased susceptibility of +/*fa* rats to DMBA-induced mammary carcinogenesis might be at least partly associated with higher leptin levels at the initiation stage. Hyperleptinemia in juvenile stages of +/*fa* rats gradually normalized and no difference in serum leptin level was found at the terminal sacrifice between the genotypes.

Histopathologically, adenocarcinomas in +/*fa* rats were more likely to present characteristic features such as moderate/poor differentiation, nuclear atypia, prominent interstitial cell proliferation and low ER α positivity. Expression levels of aromatase were decreased in mammary carcinomas regardless of the phenotype as compared to normal mammary tissue. On the other hand, leptin receptor and leptin were expressed with various intensities and no distinct differences were found between carcinomas with and without atypia. Although the cause of the lowered ER α protein expression in the moderately/poorly differentiated carcinomas with atypia is not clear, one possibility is that mammary epithelial cells of +/*fa* rats were initiated under conditions without estrogen-dependence but with close dependence on other growth factors, such as leptin or EGFR (10,23). Activation of the mitogen-activated protein kinase (MAPK) system has been demonstrated in moderately/poorly differentiated carcinomas with atypia. Thus, Thordarson *et al* (24) reported that *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinomas in ovariectomized Sprague-Dawley rats showed a more aggressive phenotype with a significant increase in MAPK activity (phosphorylation) as compared to carcinomas in intact rats, suggesting a relationship between loss of estrogen-dependence and growth. Also, in an estrogen-non-responsive human breast cancer cell line, MAPK activity was found to be increased as compared to the original estrogen-dependent sample, suggesting that increased activity of MAPK may contribute to the estrogen non-responsive growth phenotype (25).

Epidemiologically, breast cancer rates among pre-and perimenopausal ages are reported to be higher among US-born Chinese than those born in foreign countries, and similar findings were found in Filipina women as well, to the extent that contemporary rates may equal or exceed those of non-Hispanic Whites, indicating that becoming acculturated to the western lifestyle might be a breast cancer risk factor to some younger Asian women (26). Plasma leptin levels were demonstrated to be twice as high in US-born South Asian (India, Bangladesh, Sri Lanka) women aged 18-30 years than in European women (27), presumably related to the increasing rate of breast cancer. In addition, in certain Asian countries, such as India and Singapore, breast cancer patients present at a younger age, with more advanced stage and fewer estrogen-ER-positive tumors, as compared to western countries (28,29). Therefore, we propose that the present DMBA-induced mammary carcinogenesis in +/*fa* lean Zucker rats may be a useful model of increasing breast cancer in younger Asian women.

A further significant finding of the present DMBA-induced mammary carcinogenesis study in +/*fa* and +/+ rats with and without 10% corn oil diet is that elevation of serum leptin level may contribute to the growth of mammary tumors. In our preliminary study, corn oil diet, similarly prepared as in the present study, significantly elevated serum leptin concentrations of 12-week-old +/+ and +/*fa* rats as compared to basal diet, as also confirmed in the present study. These data are consistent with the previous reports of overexpression of leptin and its receptor in human breast cancer cases (30,31), and in *in vitro* studies revealing that leptin can stimulate breast cancer cell proliferation (23,32). From epidemiological studies, it is well recognized that obesity increases the risk of breast cancer in postmenopausal women, with a suggested association with menstrual and reproductive factors (33) or higher circulating levels of leptin. However, the mechanisms have yet to be fully elucidated (34,35).

In conclusion, +/*fa* rats in the present study proved more susceptible to DMBA-induced mammary carcinogenesis than +/+ controls, and this might be at least partly related to the higher leptin levels in the early stages. Corn oil diet possibly contributed to the growth of mammary tumors via elevated serum leptin levels. In addition, an aggressive phenotype of carcinoma, in which MAPK cascade but not estrogen signaling was activated, was found predominantly in +/*fa* rats. Further studies are required to examine the mechanisms of MAPK activation for mammary carcinogenesis in +/*fa* rats.

Acknowledgements

The present study was supported in part by a grant-in-aid for Cancer Research (19-2) and the Third-Term Comprehensive Control Research for Cancer (H22-G-014) from the Ministry of Health, Labour and Welfare of Japan. The authors thank Dr Malcolm A. Moore for revision of the text and Ms. Ayako Kaneko and Ms. Satomi Kohno for their expert technical assistance. We also thank the National Cancer Center Research Core Facility for some of the analyses in this study. The Core Facility was supported by the National Cancer Center Research and Development Fund (23-A-7).

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The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.37, No.5, 1025-1034, 2012

Original Article

Female heterozygous (+/*fa*) Zucker rats as a novel leptin-related mammary carcinogenesis model

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(Received June 1, 2012; Accepted August 18, 2012)

ABSTRACT — The homozygous mutant fatty Zucker rat (*fa/fa*) is the prominent model for the research of obesity, one of the most well-known risk factor of postmenopausal mammary cancer. But the usage as a mammary gland carcinogenesis model is considered to be restricted due to the hypoplasia of mammary gland. In the present study, to find the validity of heterozygous mutant (+/*fa*) lean Zucker rats as a new leptin-related mammary carcinogenesis model, we examined whether the number of terminal end buds of mammary gland, the serum biochemistry, leptin concentration in serum and adipose tissue are changed in 7-week-old female +/+, +/*fa* and *fa/fa* rats, and whether these changes and leptin, TNF-α and VEGF mRNA expression in adipose tissue of +/+ and +/*fa* rats are influenced by 10% corn oil diet for 5 weeks. We confirmed that mild hyperleptinemia was more pronounced in 7-week-old +/*fa* as compared with wild type (+/+) and hypoplasia of mammary glands characterized by fewer numbers of terminal end buds in *fa/fa* was not observed in +/*fa*. With 10% corn oil diet, leptin mRNA expression in adipose tissue showed increasing tendency both in +/*fa* and +/+. Comparing with +/+, adipose tissue in +/*fa* treated with 10% corn oil diet was found to be significantly increased in the concentration of leptin protein and tended to be elevated expression of TNF-α mRNA. These results suggest that +/*fa* with 10% corn oil diet may be a useful model for investigation of the participation of leptin and TNF-α in mammary gland carcinogenesis.

Key words: Leptin, Tumor models, Mammary cancer

INTRODUCTION

General obesity is an important risk factor of mammary cancer in postmenopausal women, and central obesity was further reported to increase mammary cancer risk in premenopausal as well as postmenopausal populations (Calle and Thun, 2004; Phillips *et al.*, 1996; Schaffler *et al.*, 2007). The mechanisms involved remain largely unclear, but it is suggested that various bioactive factors synthesized by adipose tissue might exert tumor-stimulatory effects on the mammary gland epithelium (Caldefie-Chezet *et al.*, 2005; Housa *et al.*, 2006). One principal bioactive substance produced by adipocytes is leptin (Anubhuti and Arora, 2008), a 167-aminoacid peptide hormone encoded by the obesity gene (*ob*), which is secreted and plays important roles in regulating food intake and energy expenditure through binding to specific receptors (OB-R) (Anubhuti and Arora, 2008). Leptin also controls other common physiological processes such as immune responses, cell differentiation, proliferation and angiogenesis (Zhang *et al.*, 2005). Furthermore, several evidences suggest that leptin could be involved in tumorigenesis as a mitogenic, transforming or migration factor, especially active in the development of mammary, colorectal and prostate cancers (Garofalo and Surmacz, 2006; Hu *et al.*, 2002; Rouet-Benzineb *et al.*, 2004; Somasundar *et al.*, 2004).

Both leptin and OB-R appear to be significantly over-expressed in human mammary cancer tissue relative to non-cancer epithelium (Ishikawa *et al.*, 2004). In addition, higher expression of OB-R protein has been demonstrated in estrogen receptor α (ERα)-positive human mammary carcinoma cells MCF-7 and T47D than ERα-negative carcinoma cells MDA-MB-231 and MDA-MB 435 (Garofalo *et al.*, 2004). Leptin stimulates estrogen production through enhanced aromatase mRNA expres-

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sion, protein content and enzymatic activity in MCF-7, via AP-1 (Catalano *et al.*, 2003). In general, elevated lifetime estrogen exposure is considered a major risk factor for mammary cancer in human (Key *et al.*, 2002). Leptin signaling is also reported to play an important role in the growth of mammary cancers through promotion of the expression of vascular endothelial growth factor (VEGF)/vascular endothelial growth factor receptor type 2 (VEGFT2) (Rene Gonzalez *et al.*, 2009). Moreover, its synthesis is influenced most notably by tumor necrosis factor-α (TNF-α) (Zhang *et al.*, 2000), insulin (Cusin *et al.*, 1995) and reproductive hormones (Machinal-Quelin *et al.*, 2002), all of which have been associated with mammary gland neoplastic processes. For example, there is evidence that hyperinsulinemia promotes mammary cancer progression through leptin-dependent mechanisms (Bartella *et al.*, 2008; Garofalo *et al.*, 2006). Estrogen regulates leptin productions in rats and humans subjects *in vivo* (Alonso *et al.*, 2007; Shimizu *et al.*, 1997).

TNF-α is a multifunctional cytokine that plays important roles in diverse cellular events such as immune function, cell survival, proliferation, differentiation, and death (Wang and Lin, 2008). Administration of TNF-α increased leptin mRNA and protein levels in adipose tissue of hamsters (Grunfeld *et al.*, 1996). Adipose tissues of the obese *db/db, ob/ob, tub/tub* mice, and the *fa/fa* Zucker rat expressed high levels of TNF-α mRNA and circulating plasma levels of TNF-α protein significantly elevated in *db/db* mice (Hotamisligil *et al.*, 1993). TNF-α, a proinflammatory cytokine, has been shown to be synthesized and secreted from macrophage as well as adipocyte (Kern *et al.*, 1995; Weisberg *et al.*, 2003), which may be involved in inflammation-associated carcinogenesis (Balkwill, 2009).

In animal models, a higher body weight is linked with increased incidences of both spontaneous and chemically induced mammary tumors (Haseman *et al.*, 1994; Waxler *et al.*, 1953; Wolff *et al.*, 1982). Zucker rats with a homogeneous spontaneous mutation in the leptin receptor gene (*fa/fa*) (Phillips *et al.*, 1996) are known to be obese, hyperphagic and hyperinsulinemic (Bray, 1977). In contrast, lean Zucker (+/*fa* or +/+) rats show almost normal metabolic functions, and have been used as controls in various types of physiochemical and pathological experiments (Bray, 1977). The Zucker rat has been recognized as a superior model to investigate effects of obesity on chronic disease development, including cancer (Bray, 1977; de Assis *et al.*, 2006; Hakkak *et al.*, 2007), but its utility for investigations of mammary carcinogenesis is limited due to scant epithelial development in mature mammary glands of obese as compared with lean counterparts (Hu *et al.*, 2002). Since it was reported that young heterozygous lean Zucker (+/*fa*) rats demonstrate a number of differences from wild type lean Zucker (+/+) rats, e.g., higher body weights, fat cell size, inguinal fat pad weights, pad-to-body weight ratios, serum cholesterol, adipose tissue lipoprotein lipase and glycerol-3-phosphate dehydrogenase, hepatic and adipose tissue 6-phosphogluconate dehydrogenase activities and serum leptin levels (1.6 and 0.9 ng/ml in +/*fa* and +/+, respectively, (Cleary and Phillips, 1999)) (Cleary *et al.*, 1999; Heo *et al.*, 2002; Phillips and Cleary, 1994; Truett *et al.*, 1995; Zhang *et al.*, 1997), we here investigated whether they might provide the basis for a leptin-related mammary carcinogenesis model. Two independent experiments were performed. In experiment 1, serum biochemistry, histological characteristics of mammary glands and leptin levels of serum and adipose tissue in 7-week-old female +/*fa* lean Zucker rats were compared with those of *fa/fa* and +/+ siblings. In experiment 2, we tested whether 10% corn oil diet affects serum biochemistry and histological characteristics of mammary glands as well as leptin, TNF-α and VEGF mRNA expression in adipose tissue of female +/*fa* and +/+ lean Zucker rats.

MATERIALS AND METHODS

Animals

Homozygous obese (*fa/fa*), heterozygous lean (+/*fa*) and wild type (+/+) female Zucker rats at 6 weeks of age were purchased from Charles River Japan (Kanagawa, Japan). They were housed in clear polycarbonate cages with heat-treated white wood chips for bedding (Sankyo Laboratory Service, Tokyo, Japan) in an air conditioned room (24 ± 1°C, 55 ± 5% relative humidity, 12 hr light and dark cycle) and given basal diet (CRF-1, Oriental Yeast, Tokyo, Japan) and tap water *ad libitum*. The composition of the basal diet is 22.4% crude protein, 5.7% crude fat, 6.6% crude ash, 3.1% crude fiber, 7.8% moisture content and 54.5% nitrogen-free extract and the calorie of cereal-based diet is 359 kcal/100 g. The present study design was approved by the Animals Care and Utilization Committee of the National Institute of Health Sciences.

Genotyping

The animals were divided into each genotype group on the basis of genotyping, as described previously (Phillips *et al.*, 1996). For polymerase chain reaction (PCR) amplification of DNA sequences encoding leptin receptor isoform, digested 0.5 mm tail samples were amplified with the primers 5'-GTTTGCGTATGGAAGTCACAG-3' and

5'-ACCAGCAGAGATGTATCCGAG3' at the annealing temperature of 67°C for 30 cycles. The PCR products were incubated with *Msp*I for 1 hr at 37°C to indicate the presence of the mutation-derived restriction site in Zucker rat genomic DNA.

Experiment 1

Female Zucker rats (+/+, n = 8; +/*fa*, n = 16; *fa*/*fa*, n = 6) at 7-weeks of age were weighed and sacrificed without overnight fasting and blood samples were collected from the abdominal aorta under ether anesthesia for serum biochemistry and leptin and insulin enzyme assays. Serum biochemistry measurements of glucose, triglyceride (TG), total cholesterol (T-Cho) and a double antibody radioimmunoassay for estradiol were performed at SRL (Tokyo, Japan). Leptin levels in serum and homogenates of adipose tissue that carefully excluded their mammary gland from the one side of an inguinal fat pad and serum insulin levels were measured with an enzyme-linked immunosorbent assay (ELISA) kit, YK050 (Yanaihara Institute, Shizuoka, Japan) and by rat insulin ELISA (Mercodia AB, Uppsala, Sweden), respectively, according to the manufacturer's instructions. After macroscopic observation of abdominal viscera, subcutis and inguinal fat pads, liver and remaining inguinal fat pads containing mammary gland tissue were removed and fixed in 10% neutral buffered formalin for routine preparation of paraffin-embedded sections, then, hematoxylin and eosin (H.E.) staining and immunohistochemical analysis were performed. Livers were weighed before processing. The other side of inguinal fat pad in each animal was used for whole-mount preparation.

Experiment 2

Female Zucker rats at 7-weeks of age were fed basal (+/+, n = 5; +/*fa*, n = 6) or basal diet + 10% corn oil (+/+, n = 14; +/*fa*, n = 15) for 5 weeks and then sacrificed without overnight fasting in the same manner with experimental 1. In addition to the measured items in experiment 1 except serum estradiol concentration and total number of TEB in whole-mount preparation, expression of mRNAs for leptin, tumor necrosis factor-α (TNF-α), vascular endothelial growth factor A (VEGFA) and aromatase in inguinal adipose tissue was analyzed using real-time reverse transcription (RT)-PCR.

Mammary gland whole mounts and quantification

The whole-mount preparation protocol was a modification of previously described procedures (You *et al.*, 2002). Freshly dissected inguinal fat pad containing mammary glands tissues were placed flat between a pair of glass slides and fixed in 10% formalin for 24 hr, then dehydrated with 70, 95 and 100% ethanol (about 3 hr in each). The samples were defatted in acetone for approximately 12 hr and rehydrated in diluted ethanol solution ranging from 100 to 40% for 3 hr each. The samples were stained with 0.005% toluidine blue for 30 min and then dehydrated again in ethanol solution. The tissue pieces were then finally immersed in xylene for approximately 6 hr and mounted on glass slides with a mounting agent. The total numbers of terminal end buds (TEB) were counted from the distal portions of the mammary gland under a stereomicroscope, according to the criteria of Russo (Russo *et al.*, 1990).

Immunohistochemistry

The streptavidin-biotin peroxidase complex method (StreptABComplex/HRP, DAKO, Glostrup, Denmark) was used to determine the expression and localization of leptin and leptin receptors in mammary glands and inguinal adipose tissue of Zucker rats at 7 and 12 weeks of age. Polyclonal antibodies against leptin (Ob) were purchased from Santa Cruz Biotechnology (A-20; Santa Cruz, CA, USA) and used at a dilution of 1/100. A polyclonal antibody against the leptin receptor (OB-R) recognizing both wild and mutant forms was accessed from Neuromics Antibodies (Edina, MN, USA) and used at 1/1000. Antigen retrieval was performed in an autoclave for 10 min at 121°C in 10 mM citrate buffer (pH 6.0) for leptin receptors. Sections were lightly counterstained with hematoxylin for microscopic examination. Negative controls without primary antibodies were included for each antigen using serial sections.

Real-time RT-PCR

Quantitative real-time RT-PCR using an ABI Prism 7000 sequence detection system (Applied Biosystems Japan, Tokyo, Japan) was performed for leptin (*Lep*), TNF-α (*Tnf*), VEGFA (*Vegfa*) and aromatase (*Cyp19a1*). One microgram aliquots of total RNA isolated from inguinal fat pads of all rats and from ovaries and livers of basal diet +/+ as positive and negative control of aromatase, respectively, in experiment 2 using Isogen™ (Nippon Gene, Tokyo, Japan) were applied to RT with a High-Capacity cDNA Archive Kit (Applied Biosystems Japan, Tokyo, Japan) in a 100 μl total reaction volume. For real-time PCR analysis, ABI Assays-on-Demand™ TaqMan probe and primer sets from Applied Biosystems (available at https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=601267/) were employed. Real-time PCR was performed in a 50-μl reaction volume using the TaqMan probe detection

system (Applied Biosystems Japan) with specific primers, the corresponding TaqMan™ MGB probes (FAM™ dye labeled) and RT products. For the quantification of expression data, a standard curve method and normalization with a housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase were applied.

Statistical analysis

Variance in data was checked for homogeneity by Bartlett's procedure. When the data were homogeneous, one-way analysis of variance for homogeneity (ANOVA) was used. In the heterogeneous cases, the Kruskal Wallis test was applied. When statistically significant differences were indicated, the Dunnett's multiple test was employed for comparisons between groups; in experiment 1, among all groups; in experiment 2, +/+ basal diet vs. +/+ 10% corn oil, +/+ basal diet vs. +/*fa* basal diet, +/+ 10% corn oil vs. +/*fa* 10% corn oil and +/*fa* basal diet vs. +/*fa* 10% corn oil. Value are presented as means ± standard deviations or standard error. p values of less than 0.05 were considered to be statistically significant. .

RESULTS

Experiment 1

In female fatty (*fa/fa*) Zucker rats at 7-weeks old of age, body weights and absolute and relative liver weights were higher ($p < 0.05$ or 0.01) than those of lean +/+ and/or +/*fa* rats (Table 1) and excess accumulation of adipose tissue in abdominal viscera, subcutis and inguinal fat pads at necropsy and increased storage of hepatocellular glycogen on histopathology were observed. There were higher ($p < 0.05$ or 0.01) concentrations of serum TG, T-Cho and insulin in *fa/fa* rats than in +/+ and/or +/*fa* rats, but not of glucose and estradiol (Table 2). No difference of body and liver weights and serum T-Cho, TG and insulin values were observed between +/+ and +/*fa* rats. Leptin concentrations in serum and adipose tissue were higher ($p < 0.05$ or 0.01) in *fa/fa* rats than +/+ and +/*fa* rats, and those of serum were also higher in +/*fa* rats ($p < 0.05$) than in +/+ rats (Table 3).

With inguinal mammary gland whole mounts, poorly developed tissue characterized by thinner ducts and immature glands and lower ($p < 0.01$) numbers of TEB (Fig. 1) was observed in female fatty (*fa/fa*) Zucker rats at 7-week old of age, as compared with lean +/+ and +/*fa* rats. Immunohistochemical analysis revealed that adipocytes in inguinal fat pad express leptin and its intensity was increased in hypertrophied adipocytes of *fa/fa* rats as compared with +/+ and +/*fa* (data not shown). Ductal and glandular epithelium of mammary gland of all genotypes showed positive reaction to an anti-leptin receptor antibody that recognized both wild and mutant form (data not shown).

Table 1. Experiment 1; body and liver weights

Genotype	N	Body weight (g)	Absolute liver weight (g)	Relative liver weight (g/100g b.w.)
+/+	8	152 ± 3[a]	7.1 ± 0.4	4.7 ± 0.2
+/*fa*	16	153 ± 8	7.2 ± 1.0	4.7 ± 0.5
fa/fa	6	222 ± 9**,##	11.5 ± 1.3**,##	5.2 ± 0.4#

N: No. of animals. [a]: Mean ± S.D.
**: Significantly different from +/+ at $p < 0.01$ (Dunnett's multiple test).
#,##: Significantly different from +/*fa* at $p < 0.05$ and 0.01, respectively (Dunnett's multiple test).

Table 2. Experiment 1; serum biochemistry

Genotype	N	Glucose (mg/dl)	Triglyceride (mg/dl)	Total cholesterol (mg/dl)	Insulin (ug/l)	Estradiol (pg/ml)
+/+	8	142 ± 7[a]	153 ± 44	90 ± 11	1.2 ± 0.3	34 ± 30[b]
+/*fa*	16	149 ± 28	165 ± 50	87 ± 7	1.4 ± 0.6	24 ± 13[c]
fa/fa	6	155 ± 40	302 ± 134#	129 ± 14**,##	6.8 ± 6.0**,##	19 ± 10

N: No. of animals. [a]: Mean ± S.D. [b]: n = 7 [c]: n = 15
**: Significantly different from +/+ at $p < 0.01$ (Dunnett's multiple test)
#,##: Significantly different from +/*fa* at $p < 0.05$ and 0.01, respectively (Dunnett's multiple test)

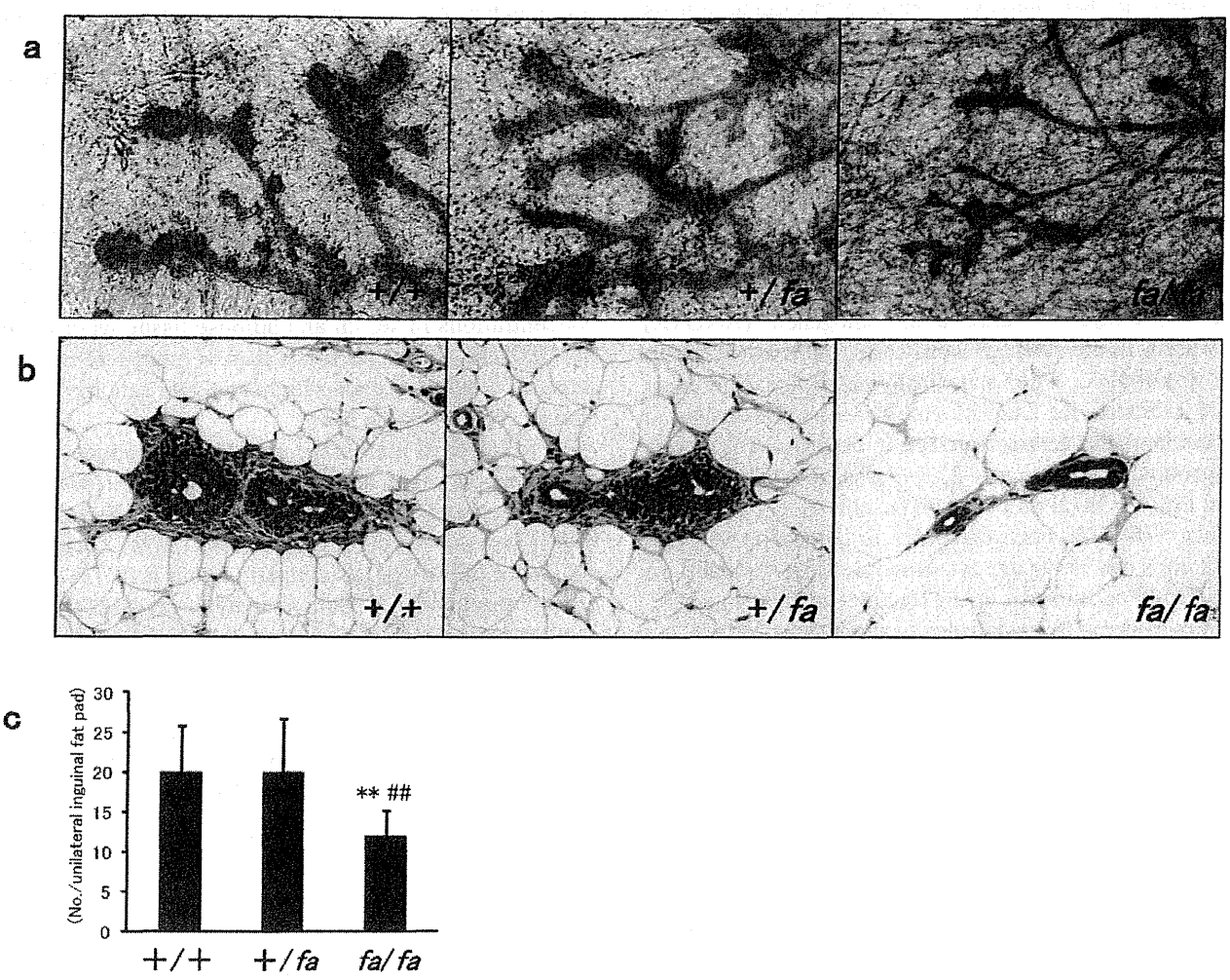

Fig. 1. Experiment 1; representative whole-mount preparation (a, toluidine blue staining, original magnification x40), histology (b, HE staining, original magnification x200) and numbers of terminal end buds (TEBs) (c) of mammary tissue of 7-week-old female Zucker rats. Poorly developed tissue characterized by thinner ducts and immature glands and lower numbers of TEB was noted in female fatty (*fa/fa*, n = 6) Zucker rats at 7-week old of age, comparing with +/+ (n = 8) and +/*fa* (n = 16) rats. **: Significantly different from +/+ at $p < 0.01$. ##: Significantly different from +/*fa* at $p < 0.01$.

Table 3. Experiment 1; leptin levels in serum and adipose tissue

Genotype	N	Serum leptin (ng/ml)	Adipose tissue leptin (ng/g)
+/+	8	0.08 ± 0.02[a]	1.3 ± 0.6
+/*fa*	16	0.14 ± 0.05*	1.6 ± 1.0
fa/fa	6	1.24 ± 0.13**,#	8.5 ± 1.6**,##

N: No. of animals. [a]: Mean ± S.D.
*, **: Significantly different from +/+ at $p < 0.05$ and 0.01, respectively (Dunnett's multiple test)
#, ##: Significantly different from +/*fa* at $p < 0.05$ and 0.01, respectively (Dunnett's multiple test)

Experiment 2

In +/*fa* rats significantly higher final body weight ($p < 0.01$) was shown in rats fed 10% corn oil when compared with rats fed basal diet (Table 4). In +/*fa* rats fed 10% corn oil, absolute and relative liver weight were significantly lower than those of +/+ ($p < 0.05$ or 0.01, Table 4). No histopathological differences in liver and mammary glands were observed between +/+ and +/*fa* rats fed basal or 10% corn oil mixed diet for 5 weeks (data not shown). In serum biochemistry, glucose concentration ($p < 0.01$) showed significantly lower values in +/*fa* rats as compared to +/+, but TG showed lower values without statis-

Table 4. Experiment 2; final body and liver weights

Genotype and diet		N	Body weight (g)	Liver weight (g)	Relative liver weight (g/100g b.w.)
+/+	Basal diet	5	229 ± 10[a]	8.2 ± 1.0	3.6 ± 0.3
	10% Corn oil diet	6	233 ± 12	8.4 ± 0.7	3.6 ± 0.2
+/*fa*	Basal diet	14	222 ± 10	7.7 ± 0.6	3.5 ± 0.2
	10% Corn oil diet	15	235 ± 9[SS]	7.8 ± 0.2[##]	3.3 ± 0.1[#]

N: No. of animals. [a]: Mean ± S.D.
[#,##]: Significantly different from +/+ 10% Corn oil diet at $p < 0.05$ and 0.01, respectively (Dunnett's multiple test)
[SS]: Significantly different from +/*fa* basal diet at $p < 0.01$ (Dunnett's multiple test)

Table 5. Experiment 2; serum biochemistry

Genotype and diet		N	Glucose (mg/dl)	Triglyceride (mg/dl)	Total cholesterol (mg/dl)	Insulin (ug/l)
+/+	Basal diet	5	179 ± 24[a]	346 ± 90	92 ± 20	1.9 ± 1.1
	10% Corn oil diet	6	172 ± 15	233 ± 77	98 ± 6	1.9 ± 0.8
+/*fa*	Basal diet	14	153 ± 13**	239 ± 100	86 ± 11	2.0 ± 0.9
	10% Corn oil diet	15	162 ± 15	176 ± 85	84 ± 10	1.7 ± 0.9

N: No. of animals. [a]: Mean ± S.D.
**: Significantly different from +/+ basal diet at $p < 0.01$ (Dunnett's multiple test)

Table 6. Experiment 2; leptin levels in serum and adipose tissue

Genotype and diet		N	Serum leptin (ng/ml)	Adipose tissue leptin (ng/g)
+/+	Basal diet	5	0.2 ± 0.1[a]	3.8 ± 1.0
	10% Corn oil diet	6	0.5 ± 0.3	2.6 ± 0.7
+/*fa*	Basal diet	14	0.3 ± 0.2	4.7 ± 2.2
	10% Corn oil diet	15	0.5 ± 0.3*	7.7 ± 1.5*,[##]

N: No. of animals. [a]: Mean ± S.D.
*: Significantly different from +/*fa* basal diet at $p < 0.05$ (Dunnett's multiple test)
[##]: Significantly different from +/+ 10% Corn oil diet at $p < 0.01$ (Dunnett's multiple test)

tical significance in +/*fa* rats with and without 10% corn oil mixed feeding as compared to their +/+ counterparts (Table 5). Insulin concentrations showed similar values among all the groups, but T-Cho concentration showed lower in +/*fa* than +/+ with 10% corn oil feeding (Table 5). Without 10% corn oil feeding, serum and adipose tissue leptin levels in +/*fa* showed a non-significant tendency for elevation than in +/+. With 10% corn oil feeding, serum and adipose tissue leptin levels were significantly increased in +/*fa* than in +/*fa* with basal diet ($p < 0.05$). Moreover, 10% corn oil feeding increased adipose tissue leptin level in +/*fa* than in +/+ ($p < 0.01$, Table 6).

With real-time RT-PCR analysis in adipose tissue, increased tendencies for leptin and TNF-α mRNA expression and opposite decrease of VEGFA were observed with the 10% corn oil diet in both genotypes (Fig. 2). Regarding TNF-α mRNA expression, +/*fa* rats showed higher tendencies regardless of the diet (Fig. 2b). Expression of aromatase mRNA was detected in ovaries as a positive control, but not in adipose tissue and livers of either +/*fa* and +/+ rats (data not shown).

DISCUSSION

The present investigation of female heterozygous lean Zucker rats in comparison with *fa*/*fa* and +/+ animals pointed to the potential of heterozygous lean Zucker rat as a possible new leptin-related mammary carcinogenesis

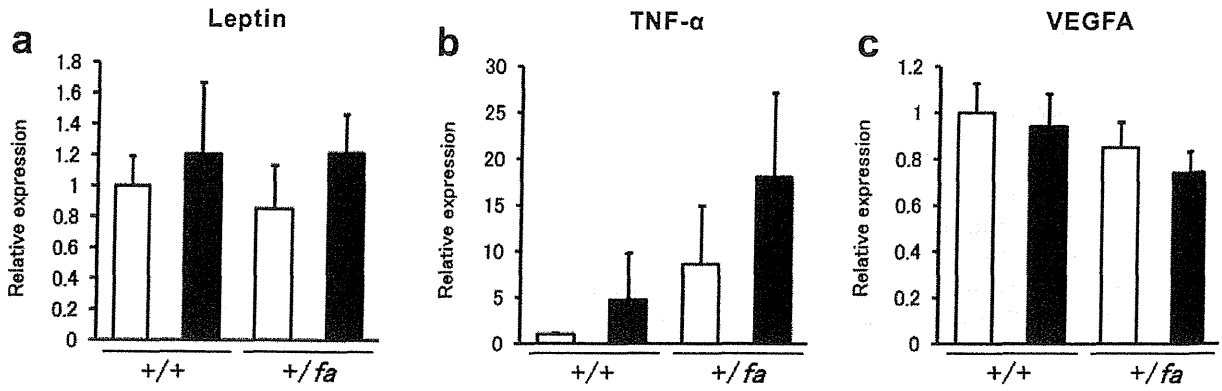

Fig. 2. Experiment 2; leptin (a), TNF-α (b) and VEGFA (c) mRNA expression in adipose tissue of female Zucker rats fed 10% corn oil for 5 weeks. Tendencies for slight increase of leptin and TNF-α expression and decrease of VEGFA were observed on feeding the 10% corn oil diet with both genotypes, but TNF-α expression showed higher tendencies in +/*fa* rats regardless of the diet. mRNA expression was normalized to the expression level of a housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase. Basal diet = open bar, 10% corn oil = closed bar. n = 3-5 (basal diet, +/+), 4-6 (10% corn oil, +/+), 10-14 (basal diet, +/*fa*), 14-15 (10% corn oil, +/*fa*). Values were set at 1 in +/+ basal diet group and expressed as mean ± S.E. relative values.

model. While fatty *fa/fa* rats show dramatically high values for serum insulin, and serum and adipose tissue leptin, they have only few TEBs at around 7 weeks of age, when rats are reported to be most sensitive to carcinogens targeting the mammary gland (Russo *et al.*, 1979). In contrast, lean +/*fa* rats feature normal mammary gland development. Corn oil-supplemented diet increased the serum leptin level. Interestingly, the increase of the leptin level in adipose tissue by 10% corn-oil diet was only observed in +/*fa* rat. TNF-α mRNA expression in+/*fa* was higher than in +/+ and further increased with corn-oil diet. All these results suggested female lean +/*fa* rats may be a potential model for investigation of mammary carcinogenesis in which leptin and TNF-α are the major related factors.

Epidemiologically mammary cancer has been shown to be associated with obesity in postmenopausal women (Calle and Thun, 2004) and hyperleptinemia is also recognized as a risk factor (Wu *et al.*, 2009). Fatty Zucker rats with hyperleptinemia have been widely used as animal obesity model which mimics human obesity and the metabolic syndrome, and recently mammary gland carcinogenicity was investigated with 7,12-dimethylbenz(*a*) anthracene (DMBA) or *N*-methyl-*N*-nitrosourea (MNU)-treated Zucker rat models. Hakkak *et al.* (2005, 2007) reported that DMBA administration by gavage at 50 days old at 65 mg/kg body weight caused more mammary tumors in female obese Zucker (*fa/fa*) rats than their lean (+/*fa* or +/+) siblings. In this model, it is uncertain which obese-related parameters, e.g., hyperinsulinemia, hyperleptinemia or hyperlipidemia, affected the mammary carcinogenesis. In contrast, Lee *et al.* (2001) indicated that no increase in susceptibility with MNU at doses of 37.5 or 20 mg/kg body weight administered to fifty-day-old female lean (+/*fa* or +/+) or obese Zucker (*fa/fa*) rats. The controversial results may be partially due to the dose of administered carcinogen based on the body weight and poor development of mammary glands with low numbers of TEBs in homozygous obese Zucker rats, as shown in Fig. 1. Scant epithelial development in mammary glands were also known in non-transgenic genetically obese leptin-deficient (*ob/ob*) and genetically obese leptin receptor-deficient (*db/db*) mice as compared with their lean counterparts (Hu *et al.*, 2002). Impaired development of mammary glands have been described in transgenic *TGF-α/ob/ob* mice (Cleary *et al.*, 2003) and high fat diet-dependent nulliparous nonpregnant obese mice (Kamikawa *et al.*, 2009). Leptin-dependent inhibition of cell proliferation has been reported in noncancerous mouse mammary epithelial cell line (Baratta *et al.*, 2003; Motta *et al.*, 2007).

It has been also reported that the reason for the poorly developed mammary gland in obese might be abnormal endogenous steroid production rather than hyperleptinemia (Marin Bivens and Olster, 1997). *fa/fa* zucker rats also show delayed vaginal opening, subsequent abnormal estrous cyclicity, undeveloped uteri and lack of deciduomata formation (Saiduddin *et al.*, 1973) as well as

abnormal estrous cycles (Marin Bivens and Olster, 1997). These facts suggest that young *fa/fa* zucker rats may have disadvantage as a mammary carcinogenesis model in aspect s of abnormal development of mammary glands and hormone environment. Therefore, the model in which the level of leptin can be effectively controlled by some exogenous factor such as diet might be a better one. In the present study, the increase of adipose tissue leptin by corn-oil diet was more evident in +/*fa* than +/+. Maher *et al.* (1996) also showed that adipose tissue leptin levels was significantly higher in fat pads of +/*fa* compared to wild type rats and +/*fa* rats fed high-fat diet showed an additional two-fold increase in leptin levels compared to wild type rats on the same diet.

It was reported that adipose tissues of the obese *fa/fa* Zucker rat expressed high level of TNF-α mRNA as compared to lean +/+ or +/*fa* (Hotamisligil *et al.*, 1993). In the present study expression of TNF-α mRNA in the adipose tissue tended to be higher in +/*fa* Zucker rats than +/+ and further increased by the 10% corn oil diet, but there was no statistical significances presumably due to small number of +/+ and/or wide variability. TNF-α stimulates the release of preformed leptin from human mature adipocytes and differentiated preadipocytes (Zhang *et al.*, 2000), and has the ability to promote tumor progression and cancer cell dissemination (Montesano *et al.*, 2005). TNF-α is synthesized and secreted from macrophage as well as adipocyte (Kern *et al.*, 1995; Weisberg *et al.*, 2003). Mammary glands of a diet induced obese mouse model harbored more infiltrating macrophages (Kamikawa *et al.*, 2009), while in the present study, increased macrophage infiltration was not observed in +/*fa* with or without the 10% corn oil diet (data not shown). Taken together, our results suggest that TNF-α expression may be stimulated by leptin-rich adipocyte rather than macrophage around mammary glands in +/*fa* and further by the 10% corn oil diet to promote leptin secretion and mammary carcinogenesis. There were no significant differences in VEGFA mRNA expression adipose tissue between our +/*fa* and +/+ rats and links with leptin gene expression are not clear (Hausman and Richardson, 2004).

Expression level of leptin mRNA in adipose tissue of 12-week-old +/*fa* rats did not show statistically significant differences from those of +/+ rats (Fig. 2a), while that of leptin protein in adipose tissue of +/*fa* fed with 10% corn oil was significantly higher than those of +/+ rats (Table 6). Our present data suggested that differences in translation efficiency, stability and efficient usage of leptin protein might be related to inconsistency of leptin mRNA expression and protein levels in +/*fa* rats. In addition, the adipose tissue leptin level in 12 week-old females +/*fa* is over 3 times higher than those of 7-week-old +/+ in the present study. A previous study indicated that adipose tissue mRNA levels for leptin were higher in +/*fa* rats than +/+ rats at 10-days of age (Zhang *et al.*, 1997). Leptin level in serum and leptin mRNA expression in adipose tissue in Wistar rats was increased with age (Oliver *et al.*, 2001).

Fasting serum glucose, TG and insulin were not changed in +/*fa* compared to +/+ in many studies (Phillips and Cleary, 1994; Schwarzer *et al.*, 1997; Zhang *et al.*, 1997). Glucose and TG concentration showed lower values with and without statistical significance, respectively, in experiment 2, but not in experiment 1. Conflicting results may be partially explained by that animals were sacrificed without overnight fasting, because serum glucose and TG levels are easily affected by food consumption. On the other hand, T-Cho concentration showed lower in +/*fa* than +/+ with 10% corn oil, which may be due to cholesterol elimination promoted by increased leptin (VanPatten *et al.*, 2001). Period of 5 weeks for diet fat supplementation is apparently shorter than that necessary for mammary carcinogenesis, but it is considered enough to examine the effects of high fat diet on the factors related to mammary carcinogenesis (Flachs *et al.*, 2006).

In conclusion, these results suggests that +/*fa* rats may be a useful model for investigation of mammary carcinogenesis in which leptin and TNF-α are the major related factors.

ACKNOWLEDGMENTS

This work was supported in part by the Grant-in-Aid for Cancer Research (19-2) from the Ministry of Health, Labour and Welfare of Japan.

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Genetic reconstitution of tumorigenesis in primary intestinal cells

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Edited by Bert Vogelstein, Johns Hopkins University, Baltimore, MD, and approved May 28, 2013 (received for review December 17, 2012)

Animal models for human colorectal cancer recapitulate multistep carcinogenesis that is typically initiated by activation of the Wnt pathway. Although potential roles of both genetic and environmental modifiers have been extensively investigated in vivo, it remains elusive whether epithelial cells definitely require interaction with stromal cells or microflora for tumor development. Here we show that tumor development could be simply induced independently of intestinal microenvironment, even with WT murine primary intestinal cells alone. We developed an efficient method for lentiviral transduction of intestinal organoids in 3D culture. Despite seemingly antiproliferative effects by knockdown of adenomatous polyposis coli (*APC*), we managed to reproducibly induce *APC*-inactivated intestinal organoids. As predicted, these organoids were constitutively active in the Wnt signaling pathway and proved tumorigenic when injected into nude mice, yielding highly proliferative tubular epithelial glands accompanied by prominent stromal tissue. Consistent with cellular transformation, tumor-derived epithelial cells acquired sphere formation potential, gave rise to secondary tumors on retransplantation, and highly expressed cancer stem cell markers. Inactivation of *p53* or phosphatase and tensin homolog deleted from chromosome 10, or activation of *Kras*, promoted tumor development only in the context of *APC* suppression, consistent with earlier genetic studies. These findings clearly indicated that genetic cooperation for intestinal tumorigenesis could be essentially recapitulated in intestinal organoids without generating gene-modified mice. Taken together, this in vitro model for colon cancer described herein could potentially provide unique opportunities for carcinogenesis studies by serving as a substitute or complement to the currently standard approaches.

colon carcinogenesis | shRNA | primary culture | Matrigel | validation

Accumulation of multiple genetic alterations underlies colon carcinogenesis, in which inactivation of adenomatous polyposis coli (*APC*) is an initiating event leading to the development of adenoma in most sporadic cases (1). Both *APC* inactivation and an activating mutation in the *CTNNB1* gene encoding β-catenin result in β-catenin accumulation through inhibition of its degradation, leading to constitutive activation of the Wnt pathway that is transcriptionally regulated by the β-catenin/transcription factor 4 (TCF) complex (2).

Widely used animal models for colorectal cancer (CRC) recapitulate tumor development in a similar manner. One is a mouse genetic model with a mutant allele of *APC*. Typically, multiple adenomas spontaneously develop predominantly in the small intestine through inactivation of the remaining allele (3, 4). The other is a chemically induced carcinogenesis model. Administration of azoxymethane (AOM) or a dietary carcinogen, 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP), recapitulates colon carcinogenesis in rodents by introducing an activating mutation in *CTNNB1* (5, 6) or inactivating *APC*, by mutation (7) or post-transcriptional down-regulation by overexpressed staphylococcal nuclease and tudor domain containing 1 (*SND1*) (8). Potential roles of genetic or environmental factors have been extensively investigated with these models. For instance, disruption of *p53* (9, 10) or phosphatase and tensin homolog deleted from chromosome 10 (*PTEN*) (11, 12) or induction of oncogenic *Kras* (13–15) significantly promoted intestinal tumorigenesis only in the context of *APC* loss. Protumorigenic effects by active inflammation have been demonstrated by inducing colitis with dextran sodium sulfate (DSS) (16). Conversely, critical roles of microflora and basal inflammation underlying tumorigenesis were also demonstrated by genetic ablation of *Myd88* (17) and *STAT3* (18), key genes in the innate immunity and inflammation, respectively.

Recent genomic and expression profile analyses have revealed a huge number of genes with mutation, deletion, or aberrant expression in human CRC (19, 20). Forward genetic screens in mice have also identified a number of genes potentially involved in intestinal tumorigenesis (21). Candidate genes for CRC have been usually validated through generation of gene-modified mice. However, it might be unrealistic to take this approach for very many genes, given the amount of time and work required for the analysis of each gene. This situation is especially true if generation of conditional KO mice and intercrossing between multiple strains becomes necessary. Alternatively, functional analyses of the genes have been widely conducted in colon cancer cell lines and fibroblasts to investigate the relevance in tumor progression and to determine oncogenic potential, respectively. However, the results might not be directly extrapolated to early stages of intestinal tumorigenesis, underscoring the definite requirement for simple validation methods in normal intestinal cells.

Given that intestinal stem cells efficiently give rise to adenoma on activation of the Wnt pathway in vivo (22, 23), we postulated that a similar approach might induce tumor development in vitro, although the requirements of intestinal microenvironment remained elusive. With recent advances in long-term culture of intestinal stem cells (24), we set out to suppress *APC* in intestinal organoids with a lentivirus. We generated tumors from intestinal organoids, independently of the in vivo setting and without using gene-modified mice. Representative genetic cooperation for tumorigenesis could be recapitulated by taking this approach, likely establishing an in vitro model for CRC.

Results

Lentivirus-Based Efficient and Stable Gene Delivery to Intestinal Organoids. To reconstitute tumorigenesis in vitro, stem cells need to be stably transduced. We adopted lentiviral gene delivery for its high infection efficiency to primary cells, including quiescent stem cells (25). However, it was revealed that Matrigel inhibited viral transduction of intestinal epithelial cells (IECs) in 3D culture, despite its definite requirement for survival. To satisfy both the presence of Matrigel and accessibility to lentiviral

Author contributions: H.N. and Y.H. designed research; K. Onuma, M.O., K. Orihashi, and Y.H. performed research; M.T. contributed new reagents/analytic tools; T.I. analyzed data; and Y.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1221926110/-/DCSupplemental.

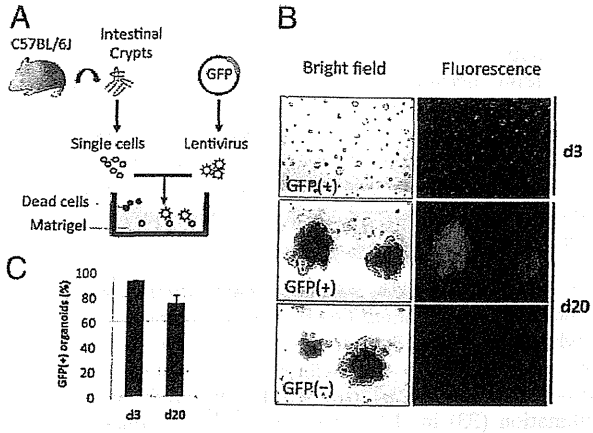

Fig. 1. Stable gene transduction of IECs in 3D culture. (*A*) Schematic diagram for lentiviral infection. Intestinal crypts isolated from C57BL/6J mice were dissociated into single cells and incubated with lentiviral particles encoding GFP for 16 h on Matrigel. (*B*) Stable and efficient transduction of organoids. Transduced organoids at day 3, at 40× magnification (*Top*). At day 20, transduced organoids consisting of GFP-positive cells (*Middle*). A non-GFP vector gave rise to only faint auto-fluorescence by dead cells at 100× magnification (*Bottom*). Representative images are shown. (*C*) Transduction efficiency to intestinal cells. Rate for GFP-positive organoids without drug selection is shown. GFP-positive and -negative organoids were counted under a microscope 48 h after the infection (day 3) or second subculture (day 20). Mean ± SD ($n = 3$) is shown.

particles, we coincubated dissociated single cells and viral particles on Matrigel (Fig. 1*A*), which achieved high transduction efficiency (Fig. 1*B*). About half of the cells composing organoids were viable, and ~20% of them attached to Matrigel with or without viral particles (Fig. S1), whereas no dead cells were observed on Matrigel. Attached cells readily developed into tiny circular organoids at day 3 (Fig. 1*B*), implying this procedure might enable preferentially capture intestinal cells of a highly proliferative nature. Even without drug selection, the GFP-positive rate was as high as 93% at day 3, which fell to 75% at day 20 (Fig. 1*C*), presumably due to slightly adverse effects by viral integration. Many organoids consisted of only GFP-positive cells at day 20, even after two rounds of subculture (Fig. 1*B*). Given the rapid turnover rate of IECs (24), we reasoned that organoids were likely reconstituted by stably transduced stem cells.

Wnt Pathway Activation in Organoids Transduced with Multiple Clones of shRNA Against *APC*. With this efficient technique, we introduced a total of five clones of potent shRNA against *APC* (shAPC) (Fig. S2*A*) individually into organoids. With a routine schedule for 3D culture (Fig. S2*B*), however, we frequently failed in propagation for any shAPC clone tested, even under drug selection (Fig. S2*C*), suggesting adverse effects by *APC* knockdown in vitro. In contrast, introduction of potent shp53 or shPTEN (Fig. S2*D*) resulted in steady propagation of organoids (Fig. 2*A*), which spontaneously became puro-resistant, suggesting a growth advantage of inactivating *p53* or *PTEN*. We later found that cointroducing all of the five shAPC clones together (hereafter referred to as shAPCs) reproducibly gave rise to rounded cystic organoids, which dominated the population over time (Fig. 2*A*; Fig. S2*C*). Similar structures have been previously documented for organoids from *APC*-deficient adenoma (26, 27), suggesting a link between the morphology and *APC* loss. However, we assumed that this might not be necessarily the case, because we knew that cystic shape could be induced independent of *APC* knockdown (e.g., under stressed culture conditions including freeze/thaw, drug selection, or too stringent dissociation), which prompted us to characterize the cystic organoids with shAPCs in more detail. We found that they were puro-resistant and indeed suppressed for expression of *APC* (Fig. S2*D*). In thin sections, they lost physiological properties such as polarity (Fig. 2*B*), differentiation (Fig. 2*C*), and cellular turnover (Fig. 2 *B* and *C*), consistent with perturbed differentiation and migration associated with *APC* inactivation (28). In addition, β-catenin accumulation indicative of Wnt pathway activation was evident (Fig. 2*D*), which was also confirmed by qPCR analysis demonstrating up-regulation of *Axin2* (Fig. 2*E*), a specific target of the β-catenin/TCF complex (29). These observations implied that the organoids with shAPCs might be essentially similar, if not identical, to those derived from *APC*-deficient adenoma.

Induction of Tumors from Organoids by RNAi-Mediated Suppression of *APC*. We next investigated whether suppression of *APC* in organoids could also lead to tumor development, as observed in adenoma in vivo. After 4 wk of culture, organoids with shAPCs corresponding to 5×10^5 cells were mixed with Matrigel and injected into nude mice. At 6 wk after injection, round and solid flesh-colored nodules frequently developed (Fig. 3*A*). They were characterized by epithelial glands and prominently infiltrated stromal cells (Fig. 3*B*). Active proliferation of epithelia was verified by high Ki-67 labeling index and inferred from β-catenin accumulation (Fig. 3*C*). Based on these features common to intestinal tumors, we classified them as "tumors." In contrast, organoids with the vector control gave rise to no nodules at all or small flat nodules with a gelatinous appearance, if any (Fig. 3*A*). As they histologically lacked epithelial glands (Fig. 3*B*), we classified them as "Matrigel plugs." In the absence of shAPCs, no tumor was induced by shp53 and/or shPTEN or from *p53*- and *PTEN*-deficient organoids (Fig. S3; Table 1), in line with earlier studies in vivo (11, 12, 30, 31). In some cases, organoids with

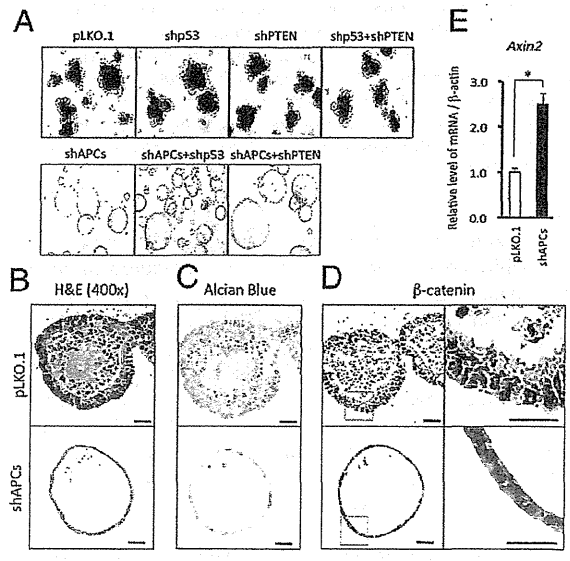

Fig. 2. RNAi-mediated suppression of *APC* in intestinal organoids. (*A*) Organoids transduced with various shRNA(s). Representative images at 4 wk after transduction are shown. Large rounded cysts were induced exclusively in the presence of shAPCs. pLKO.1 is an empty vector. (*B–D*) Transduced organoids in thin section. Serial sections were stained with H&E (*B*), Alcian blue (*C*), and β-catenin antibody (*D*). In organoids with shAPCs, intraluminal debris due to physiological turnover of intestinal cells is lost. Paneth cells stained red (*B*) and mucus stained blue (*C*) also became absent. Localization of β-catenin shifted from membrane to cytoplasm and nucleus (*D*). *Insets* in the left panel are enlarged in the right panel. (Scale bar, 20 μm.) (*E*) qPCR analysis of *Axin2* in transduced organoids. Relative expression level of mRNA to *β-actin* is shown. Mean ± SD ($n = 3$) is shown; $^*P < 0.01$.

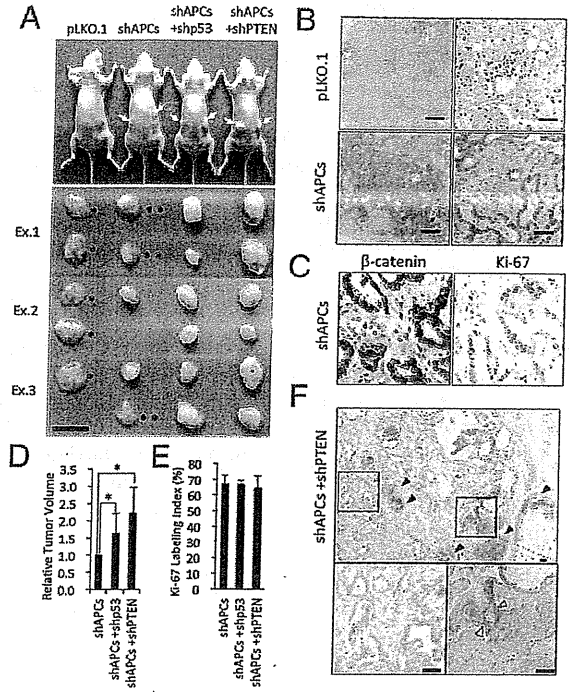

Fig. 3. RNAi-mediated induction of tumors from WT organoids. (*A*) s.c. tumors developed from injected organoids. Palpable nodules in nude mice (arrow) at 6 wk after injection (*Upper*). Excised nodules in three representative experiments (Ex.1–3). Matrigel plugs (asterisk), nontumor (double asterisks), or tumors (no asterisk) (*Lower*). (Scale bar, 10 mm.) (*B*) Histological features of the nodules. H&E staining of Matrigel plugs (*Upper*) and tumors with shAPCs alone (*Lower*) at 20× (*Left*) and 200× (*Right*) magnification. (Scale bar, 500 and 50 μm, respectively.) (*C*) Immunohistochemical analyses. Tumors with shAPCs alone stained for β-catenin (*Left*) and Ki-67 (*Right*). (Scale bar, 25 μm.) (*D*) Relative ratio of tumor volume. Mean ± SD ($n = 5$) is shown; $^*P < 0.05$. (*E*) Ki-67 labeling index for tumor epithelia. Mean ± SD ($n = 7$) is shown. (*F*) Alcian blue staining. Tumors with shAPCs+shPTEN were stained. Mucus pools stained in blue (closed arrowhead) in stroma (*Upper*). *Insets* are enlarged in lower panel. Mucus and cellular debris shed into the lumen (*Lower Left*) are leaking (open arrowhead) from the disrupted glands (*Lower Right*). (Scale bar, 50 μm.)

shAPCs alone comprised nodules resembling Matrigel plugs, but having focal white spots inside (Fig. 3*A*). We classified them as "nontumor," based on too low a proportion of epithelial cells. Thus, tumors were tentatively defined as nodules replacing coinjected Matrigel with proliferating epithelial glands at 6 wk after injection. By applying this criteria, the tumor development rate by shAPCs alone was 63% (=7/11), 5 cases for both sides and 2 cases for either side, among 11 cases (Table 1). These results suggested that *APC* suppression might be integral but not always sufficient for tumor development from organoids, consistent with earlier studies in vivo (17, 18, 32).

Suppression of *p53* or *PTEN* Promotes *APC*-Dependent Tumorigenesis from Organoids. Many gene-modified mice have been crossed with *APC* mutant mice to evaluate their impact on carcinogenesis, in which common readouts were multiplicity, size, and histology of the tumors. We wondered if similar analysis could be feasible at the cellular level. By cointroducing shp53 or shPTEN with shAPCs into organoids (Fig. 2*A*), tumor development was observed for both sides of nude mice in all of the cases tested (Fig. 3*A*; Table 1). Similar results were obtained by introduction of shAPCs into *p53*- (Fig. S3) and *PTEN*-deficient organoids (Table 1). A significant increase in tumor size was also observed (Fig. 3*D*), but an increase was not observed in proliferation index (Fig. 3*E*). No remarkable effects were detected in histological features, including mucus pool formation (Fig. 3*F*), tumor gland morphology (Fig. S4*A*), and β-catenin accumulation (Fig. S4*B*). Taken together, cooperation for tumorigenesis between *APC* loss and inactivation of either *p53* (9, 10) or *PTEN* (11, 12) could be recapitulated in tumors as an increase in size and development rate. We also verified that organoid culture was conducted in a stromal cell–free condition (Fig. S5), confirming tumorigenesis was indeed achieved with IECs alone.

Significant Acceleration of *APC*-Dependent Tumorigenesis from Organoids by *Kras* Activation. To reconstitute somatic mutation of *Kras*, which is frequent in human CRC (19), we deleted a stop codon flanked by two loxP elements [Lox-Stop-Lox (LSL)] blocking the expression of $Kras^{G12D}$ by lentiviral Cre-mediated recombination (33) in IECs from $Kras^{LSLG12D/+}$ mice (34, 35). Successful deletion was confirmed by detecting the "1-loxP" fragment (35) in genomic PCR (Fig. 4*A*). Amplification of the LSL cassette revealed its partial and complete deletion in organoids with Cre and shAPCs+Cre, respectively (Fig. 4*A*). On $Kras^{G12D}$ expression, active Ras enriched (Fig. 4*B*) without affecting the morphology of the organoids (Fig. 4*C*), verifying specific activation of Ras but not the Wnt pathway. We then asked whether a synergy between oncogenic *Kras* and *APC* loss in intestinal tumorigenesis (13–15) could be recapitulated in our model. Strikingly, organoids with shAPCs+Cre gave rise to tumors on both sides so rapidly that the nude mice became moribund at 2 wk after injection (Fig. 4*D*) in 11 of 11 cases (Fig. 4*E*). They typically appeared red, indicative of active angiogenesis and hemorrhage, and contained cystic dilatation due to retention of serous fluid (Fig. 4*D*). Compared with organoids with shAPCs alone, a significant increase in tumor size was observed (Fig. 4*F*). Cre did not synergize with shAPCs in $Kras^{+/+}$ organoids (Fig. S6), ruling out the possibility of direct synergy between Cre and shAPCs. Tumor glands became more densely packed with morphological alteration from an irregular cystic structure (Fig. 5 *B* and *E*) to a tubular or papillary structure (Fig. 5 *C* and *F*). Destruction of glands leading to mucus pool formation (Fig. 5*H*) disappeared, despite retained mucus production ability (Fig. 5*I*). Given no effects on both cell proliferation (Figs. 4*G* and 5 *K* and *L*) and the magnitude of β-catenin accumulation (Fig. 5 *N* and *O*), $Kras^{G12D}$ might have induced tumor growth through histological alterations. Thus, the synergy was successfully recapitulated in tumors as an increase in size and development rate and alteration in histology.

Marginal Effects by *Kras* Activation Alone on Tumorigenesis from Organoids. We also characterized nodules with either of shAPCs or $Kras^{G12D}$ at 2 wk postinjection for reference, although this was too early for the correct diagnosis. If the criteria for tumors were automatically applied, tumor-positive cases were seven of seven for shAPCs, three of seven for $Kras^{G12D}$, and zero of seven for pLKO.1 (Fig. 4*E*). Putative tumors induced by $Kras^{G12D}$ contained

Table 1. Summary of tumor development induced by shRNA transduction

Genes/genotypes		V	P	5	5P	A	A5	AP	A5P
shRNA	shAPCs					+	+	+	+
	shp53			+	+		+		+
	shPTEN		+		+			+	+
	pLKO.1	+							
IEC	WT	0/14	0/4	0/5	0/3	7/11	8/8	10/10	1/1
	p53 $^{-/-}$	0/4	0/2	—	—	2/2	—	4/4	—
	PTEN $^{-/-}$	0/1	—	—	—	—	1/1	—	—

—, not tested. V, P, 5, and A depict vector, shPTEN, shp53, and shAPCs, respectively.

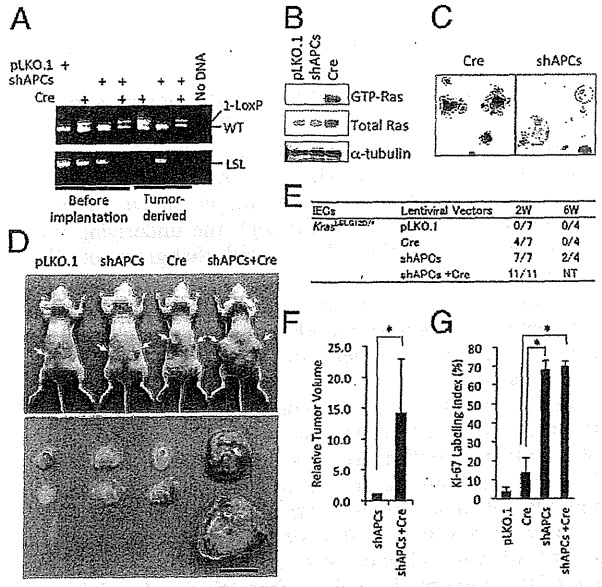

Fig. 4. Synergy between *APC* suppression and *Kras* activation in organoids for tumorigenesis. (*A*) Cre-mediated recombination in vitro. Genomic PCR analysis for WT and recombined allele of *Kras* (*Upper*) and for the LSL cassette (*Lower*). 1-LoxP, single LoxP after the recombination. (*B*) Enrichment of active Ras by induction of $Kras^{G12D}$. Immunoblotting analysis for Ras before (*Middle*) and after (*Top*) GST pulldown assay. α-Tubulin serves as a loading control (*Bottom*). (*C*) Transduced organoids in 3D culture. $Kras^{G12D}$ did not induce cystic shape. (*D*) s.c. tumors in nude mice. Palpable nodules (arrow) at 2 wk postinjection (*Upper*) and corresponding nodules after excision (*Lower*). (Scale bar, 10 mm.) (*E*) Summary of tumor development. Data at 2 and 6 wk after the implantation are shown. NT, not tested. (*F*) relative ratio of tumor volume. Mean ± SD ($n = 7$ each) is shown, $*P < 0.01$. (*G*) Ki-67 labeling index for epithelia in the nodules. Mean ± SD is shown; $*P < 0.01$. pLKO.1 ($n = 3$), Cre ($n = 3$), shAPCs ($n = 7$), and shAPCs+Cre ($n = 7$).

a few glands of ductal or cystic shape (Fig. 5 *A* and *D*), with impaired cell differentiation and proliferation as exemplified by loss of mucus production (Fig. 5*G*) and low Ki-67 index (Figs. 4*G* and 5*J*), respectively. Consistent with the lack of Wnt pathway activation (Fig. 4*C*), β-catenin remained in the membrane (Fig. 5*M*). $Kras^{G12D}$ nodule-derived organoids proved completely deleted for LSL, which had been only partially deleted when injected (Fig. 4*A*), suggesting their transient growth advantage. However, $Kras^{G12D}$ tumors no longer remained at 6 wk after injection (Fig. 4*E*), indicating that *Kras* activation by itself was insufficient for establishment of tumors, consistent with previous studies reporting no effect in the small intestine (13, 14, 36) and induction of only hyperplasia in the colon (15, 37). Also, initial proliferation and eventual extinction might mirror the natural course of aberrant crypt foci (ACF) (38), which are early lesions of the colon highly associated with *Kras* mutation (39).

Acquired Cancer Stem Cell-Like Properties in Tumor-Derived Organoids. As intestinal stem cells (ISCs) were unable to survive in s.c. tissue (Fig. 3*A*), development and maintenance of tumor glands with differentiated and proliferative properties (Fig. 3 *C* and *F*) suggested the emergence of a distinct subpopulation with the ability to self-renew and differentiate. To better characterize the nature of induced tumors, we harvested all of the nodules to conduct organoid cultures and obtained organonids only from tumors. *APC* and *PTEN* were suppressed by corresponding shRNAs (Fig. 6*A*), confirming successful transduction. Tumor-derived organoids proved tumorigenic in all seven cases examined. Notably, tumors from identical cells gave rise to secondary tumors akin to the primary tumors in both magnitude (Fig. 6*B*) and histology (Fig. S7*A*), regardless of genetic background (Fig. S7*B*), further implying the emergence of a cancer stem cell (CSC)-like subpopulation. Sphere-forming potential in suspension culture has been associated with stemness (40). Whereas single cells containing ISCs did not form spheres, tumor-derived organoids yielded spheroids (Fig. 6*C*) in all seven cases examined. Even never-implanted organoids with shAPCs alone formed spheroids (Fig. 6*C*), suggesting induction of the CSC-like properties even before injection into nude mice. Quantitative PCR (qPCR) analysis revealed up-regulation of CSC markers *CD44* and *CD133* (41) but not ISC markers *Lgr5* or *Bmi1* (42) in tumor-derived organoids (Fig. 6*D*). Despite up-regulation of *Axin2* and *CD44* in tumors, *c-Myc* or *CCND1* were not induced, suggesting selective activation of a subset of Wnt target genes toward acquisition of CSC properties. Taken together, these results supported the notion that ISC-containing organoids likely comprised a subpopulation with CSC-like properties through *APC* inactivation.

Discussion

To model human CRC, inactivation of *APC* and subsequent tumor development in the intestine have basically been achieved in mutant or gene-modified mice for *APC* (4). In contrast, we demonstrated that it could also be achieved without a genetically

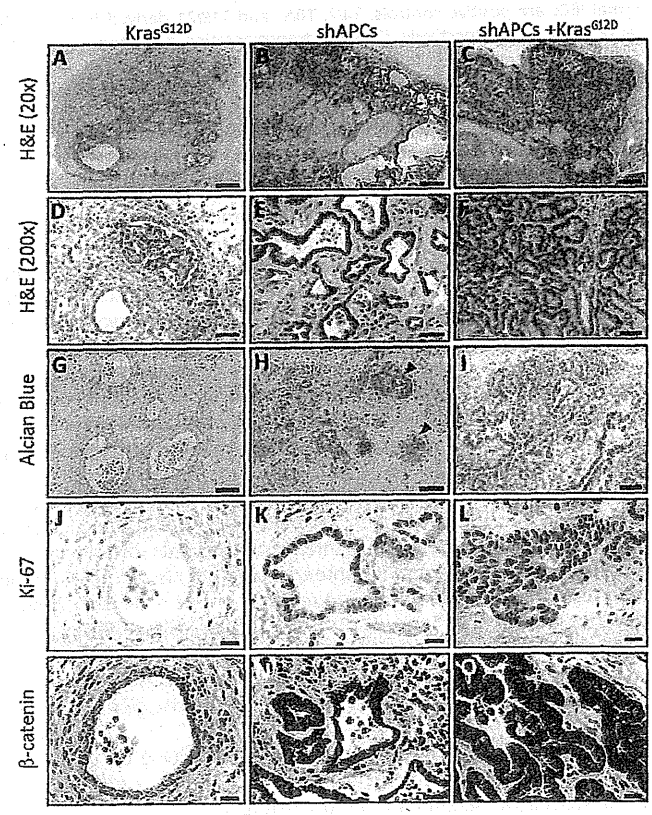

Fig. 5. Histological features of the tumors induced from $Kras^{LSL-G12D/+}$ organoids. (*A–F*) H&E staining at 20× (*A–C*) and 200× (*D–F*) magnification. (Scale bar, 500 and 50 μm, respectively.) (*G–I*) Alcian blue staining. No mucus production (*G*), formation of multiple mucus pool (closed arrowheads) in the stroma (*H*), and mucus confined in the lumen of intact glands (*I*). (Scale bar, 100 μm.) (*J–L*) Immunostaining for Ki-67. Few (*J*) and many (*K* and *L*) positive cells in the tumor glands are observed. (Scale bar, 25 μm.) (*M–O*) Immunostaining for β-catenin. Localized in the membrane (*M*), and accumulated in the cytoplasm or nucleus (*N* and *O*). (Scale bar, 25 μm.) Representative images are shown. Tumors were generated by $Kras^{G12D}$ (*Left*), shAPCs (*Center*), or both (*Right*) from identical cells and harvested at 2 wk after injection.