Reduction of pancreatic acinar cell tumor multiplicity in Dnmt1 hypomorphic mice

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Introduction

DNA methylation patterns are frequently altered in human cancers compared with their normal counterparts. DNA methylation differences between pancreatic neoplasia and histologically normal pancreas have been reported in several human studies (1–5). However, the functional relevance of most of these DNA methylation changes is unclear. Mouse models offer an attractive approach to understand the role of DNA methylation in tumor development.

Manipulation of the levels of DNA methyltransferase (Dnmt) 1, a maintenance DNA methyltransferase, have been used as a tool to study the effect of DNA hypomethylation on tumorigenesis in several in vivo studies. Reduction of DNA methylation levels has been accomplished through the use of demethylating agents either separately or in conjunction with genetic manipulations of Dnmt1 (6–14). The role of DNA methylation in vivo was first explored in the intestine using the Apc multiple intestinal neoplasia (Min) model, a commonly used mouse model of intestinal tumors since it very closely mimics the human familial adenomatous polyposis (FAP) condition (7,15,16). These mice develop benign intestinal tumors with a rare occurrence of malignant cancers (15). Treatment of Apce−/− mice with the demethylating agent, 5-aza-2′-deoxycytidine, significantly reduces tumor formation in the intestine, suggesting that DNA methylation may play an important role during tumorigenesis (7). We have further shown that crossing Apce−/− mice with Dnmt1 hypomorphic mice results in complete suppression of macroscopic intestinal neoplasia (6). Reduced Dnmt1 expression also affects the frequency of malignant intestinal tumors in DNA mismatch repair deficient mice (Mih1−/−) (8).

The use of Dnmt1 hypomorphic alleles has allowed for viable down-regulation of Dnmt1, since complete knockout of the maintenance methyltransferase is embryonic lethal (17). In this study, we have used the N- and the R-hypomorphic Dnmt1 alleles. The Dnmt1N allele was generated by replacement of part of exon 4 by a neomycinR cassette as described by Li et al. (17). This allele has ~10% of the expression of the wild-type allele (6,8). Dnmt1R allele was constructed by insertion of three copies of the lac operator sequence (lacO) from Escherichia coli into intron 3, just upstream of the splice acceptor site as described by Eads et al. and by Trinh et al. (6.8). The R-allele is expressed at about half of wild-type levels (6.8). Mice homozygous for the Dnmt1 N-allele die at the embryonic stage E10.5 (17).

Here, we study the role of DNA methylation in pancreatic cancer. Aberrant DNA hypermethylation patterns have been observed in both early- and late-stage human pancreatic tumors (1–5). We hypothesize that reduction in DNA methylation levels may decrease pancreatic tumor burden in vivo. Mice heterozygous for mutation of Apc are predisposed to the development of benign intestinal polyps, whereas mice homozygous for a mutation in the Trp53 gene develop a wide range of malignancies, including sarcomas and lymphomas (18). The combined mutation of Apce−/− and Trp53−/− has been shown to result in a shift in phenotype with nearly 83% of the animals developing abnormalities of the exocrine pancreas, of which 22% also presented with pancreatic acinar cell carcinoma (19). To investigate the role of DNA methylation in pancreatic tumorigenesis, we have used this mouse model of exocrine pancreatic cancer and crossed it with mice carrying hypomorphic alleles of Dnmt1.

Materials and methods

Animal models

C57BL/6 Dnmt1+/− mice were provided by Dr R.Jaenisch at the Whitehead Institute for Biomedical Research. The formal nomenclature for this allele is Dnmt1Δp/Δp. 129SvJae Dnmt1+/− mice were generated as described previously (6,8). C57BL/6 Apce−/− mice were obtained from Jackson Laboratories (Bar Harbor, ME) (15). Trp53−/−deficient mice were generated and characterized by Jacks et al. in 1994. Trp53−/− mice in the 129SvJae background were provided by Dr R.Jaenisch at the Whitehead Institute for Biomedical Research. Trp53−/− mice were backcrossed to the C57BL/6 background for at least 12 generations. Mice were monitored on a daily basis for signs of illness or tumor development. All mice were treated in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Southern California (USC).

Generation of Apce−/−; Trp53−/− and Dnmt1−/− mice

The triple cross was generated by crossing 129SvJae Apce−/−, Trp53−/− and Dnmt1−/− females with C57BL/6 Apce−/−, Trp53−/− and Dnmt1−/− males, producing 24 genotypic combinations. The probability of obtaining the desired four Dnmt1 genotypes with Apce−/−; Trp53−/− is 1/8.

Genotyping of mice

DNA was isolated from ~1 cm tail biopsies of 4-week-old mice as described by Laird et al. (1991) (20). The genotype of the Dnmt1 alleles was determined by multiplexing polymerase chain reaction (PCR) primers OL106 (5 ’ GGGAATTCCTCCTGACTAGGGG 3 ’), OL168 (5 ’ CCCAACAACCCAGTATGCTCGT 3 ’), OL173 (5 ’ CCCAGTTTCCAGAAAGCTACC 3 ’) and OL369.
(5′-CAATTCCACACAACTACAGACG-3′) as described by Trinh et al. and Eads et al. (6,8). The reaction was carried out in a 30 μl volume with 1× PCR buffer without MgCl₂, 1.5 mM MgCl₂, 0.2 mM deoxynucleotide triphosphate, 0.344 μM primer mix and 0.5 μl Taq polymerase (Cat No. 10342-020; Invitrogen, Carlsbad, CA). Apc genotyping was performed using real-time PCR in a 30 μl reaction as described by Peterson et al. (21). The samples PCR primers: OL012 (5′-TTATAGGACCACGCAGGG 3′) and OL014 (5′-TCTCTGCGTCTTACAGTAC 3′) and OL012 and OL014 amplify the Trp53 wild-type allele, whereas OL013 and OL014 amplify the knockout allele. The PCR conditions are 94°C for 5 min, then 40 cycles of 94°C for 45 s followed by 55°C for 45 s and 72°C for 1 min, ending with 72°C for 5 min. The bands were resolved on 2% agarose gel where the wild-type Trp53 allele was 645 bp band and the null allele gives a 600 bp band.

Histological and β-catenin immunohistochemical analysis
Pancreas tissues from ApcMin/+; Trp53−/− mice with the different Dnmt1 combinations were collected, fixed overnight in 10% buffered formalin and stored in 70% ethyl alcohol.

Paraffin sections were stained with hematoxylin and eosin for histological examination. For immunohistochemistry, paraffin sections were deparaffinized, rehydrated; antigen retrieval was achieved in 10 mM Citrate Buffer pH 6.0. Entire lysate was bisulfite converted using the EZ DNA Methylation™ Kit (Cat. No D5002; Zymo Research Corporation, Irvine, CA.) as described by Trinh et al. (6,8). The reaction was carried out in a 30 μl volume with 1 μl of sample. The PCR buffer (100 mM Tris–HCl pH 8.0, 10mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 2 mg/ml Proteinase K (added fresh) and 0.05 mg/ml of transfer RNA.). Entire lysate was bisulfite converted using the EZ DNA Methylation™ Kit (Cat. No. D5002; Zymo Research Corporation, Irvine, CA.). The reaction was carried out overnight at 55°C. The reactions were amplified on the Opticon DNA Engine Continuous Fluorescence Detector (MJ Research/Bio-Rad, Hercules, CA). Two different probes were used to distinguish the wild-type allele from the Min-allele. Probe sequence used to amplify the Apc−/− allele was 6-FAM-TCTCTTCCACGTCATTGCG-BOHQ-1, CGAACCCGACTCTCCGAACGACGCG-BOHQ-1, ATACCGGGCATACCCCGCCA-BOHQ-1, ATACCGGGGAAACCTCGCCA-BOHQ-1, CCGCGAAGAGGTGTCG-BOHQ-1, CAGCGACCGGGATTAATGTAGC-1000, 6FAM-CAGAGGCTGCTCCTAACAGCT-BOHQ-1, ACTTCACTCTCCCGAGGATTAATGTAGC-1000, 6FAM-CCTAGAGGCTGCTCCTAACAGCT-BOHQ-1, AACCAAAATACATCCTCGAACCTCCA-BOHQ-1, CACAGCACGAGGAGGAACGAGGTGTCG-BOHQ-1, and the null allele gives a 600 bp band.

DNA isolation and bisulfite conversion
Ten micrometer sections of paraffin-embedded pancreas were cut, mounted on charged slides and stained with hematoxylin and eosin. Appropriate tissue types were microdissected and incubated overnight at 50°C in 40 μl of lysis buffer (100 mM Tris–HCl pH 8.0, 10mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 2 mg/ml Proteinase K (added fresh) and 0.05 mg/ml of transfer RNA.). Entire lysate was bisulfite converted using the EZ DNA Methylation™ Kit (Cat. No. D5002; Zymo Research Corporation, Irvine, CA.). The reaction was carried out overnight at 55°C. The bisulfite converted DNA was diluted by bringing the final volume up to 300 μl. Only 10 μl was used for each subsequent Methylation reaction.

DNA methylation analysis
Genomic DNA was isolated (see Materials and methods—DNA isolation and bisulfite conversion) and subjected to bisulfite treatment (see Materials and methods—DNA isolation and bisulfite conversion) followed by Methylation analysis. Methylation Light analysis. Methylation Light data are provided in the form of a ratio using a standard curve to extrapolate the log value using the threshold Ct (t) value between the methylation locus of interest and a methylation-independent normalization reaction. In our studies, the methylation-independent reaction was the mouse repetitive element, IAP. Also, due to variation in reaction performance and other PCR parameters, it is necessary to normalize this ratio to the ratio obtained for a constant reference sample, M.Ss SCRIPT-treated DNA. M.Ss SCRIPT-treated reference sample is also used to generate the standard curve. The values are given as percentage methylated reference (PMR), which is [100 × (methylation reaction/control reaction)]/sample/methylated reaction/control reaction] × 100%.

M.Ss treatment
Wild-type mouse tail DNA was used as template for M.Ss treatment. Tail DNA at a concentration of 0.05 μg/ml was incubated with M.Ss (Cat. No. M0226L; New England BioLabs, Ipswich, MA) at a concentration of 0.05 μg/ml and 0.16 μM S-adenosylmethionine overnight at 37°C. Two additional aliquots were added to the mixture for two consecutive days with 2× S-adenosylmethionine (0.20 mM) and M.Ss (0.05 μg/ml) and allowed to incubate again overnight at 37°C. The mixture was phenol:chloroform purified and the incubation steps above were repeated once more. The final product was denatured 2× M.Ss, indicating the tail DNA underwent two rounds of M.Ss treatment to ensure complete methylation of cytosine in the context of CpG dinucleotides.

Statistical analysis
Statistical analyses were performed using R. Trend test for tumor multiplicity was done using Poisson Regression in R. Test trend for DNA methylation was done by transforming PMR measurements to be within the [0,1] range to reflect its beta distribution nature. Beta regression was done to test for trend in DNA methylation changes across the groups using R package ‘betareg’. The parallel plot was plotted with R package ‘Lattice’. Paired t-test was used to test for null hypothesis that the true change in PMR is equal to zero. All P-values are two sided.

Results
Progeny generated from the triple-cross follow Mendelian distribution
We crossed 129SV/Jae Apc+/+, Trp53−/−, Dnmt1R/− females with C57BL/6 Apcm-/−, Trp53−/−, Dnmt1R/− males to test the effect of reduced Dnmt1 levels on pancreatic neoplasia. This triple cross produced a total of 761 mice with 24 genotypic combinations (Table I). An overall Chi-square analysis did not reveal a significant distortion of the observed genotypes from the expected distribution. However, Chi-square analysis of Dnmt1 genotypes without regard to Apc or Trp53 genotype revealed that the compound hypomorphic Dnmt1R/−/− progeny were significantly underrepresented (Table I). Nevertheless, there were sufficient numbers of all Dnmt1 genotypes among the Apc−/−, Trp53−/− progeny to address the effect of Dnmt expression levels on pancreatic acinar cell tumorigenesis (Table I).

Pathway deregulation observed in nearly all histologically abnormal lesions
Cohorts of Apc−/−, Trp53−/− mice with Dnmt1R/− (n = 13), Dnmt1R/− (n = 21), Dnmt1R/− (n = 22) or Dnmt1R/− (n = 11)
genotypes were aged up to a fixed time-point of 100 days, euthanized and analyzed for foci, adenomas and carcinomas of the pancreas. Hematoxylin and eosin staining was performed to detect aberrant histological abnormalities.

Pancreatic sections were compared with that of normal pancreas (Figure 1A). Tumors were classified as atypical foci for clusters of dysplastic pancreatic cells <0.030 mm² in area (Figure 1B). Because the atypical foci were often difficult to identify by strict morphologic criteria, nuclear β-catenin immunohistochemistry was used to determine their numbers. Adenomas were defined as larger circumscribed masses of dysplastic acinar cells (Figure 1C). Adenomas were typically >0.030 mm² in area. Carcinomas lacked defined boundaries, demonstrated loss of acinar architecture, more dysplasia and were typically larger, often visible to the naked eye (Figure 1D). The number of lesions was scored with a microscope by a single observer blinded to the genotype of the mice.

Loss of the wild-type Apc allele is seen in all Apc<sup>Min+</sup> mouse intestinal polyps (22). This loss results in the stabilization and nuclear accumulation of β-catenin. Therefore, we used β-catenin staining as a proxy for Wnt-pathway deregulation. We found β-catenin stabilization and nuclear accumulation in essentially all atypical foci, adenomas and carcinomas, consistent with activation of the Tcf/β-catenin-signaling pathway.

**Decrease in tumor multiplicity is observed with decreasing Dnmt1 levels**

We first investigated whether DNA hypomethylation has an affect on tumor multiplicity. This was performed by counting the number of lesions per mouse. All counts were performed using β-catenin immuno-stained slides. Tumor burden index was calculated by assigning an arbitrary score of ‘one’ for atypical foci, ‘two’ for adenomas and ‘three’ for carcinomas. Statistically significant decreasing trend in tumor multiplicity with diminishing Dnmt1 levels was observed (Figure 2A). Based on the data from Figure 2A, it was unclear whether the decreasing trend in tumor burden was due to any one particular lesion or whether the decreased Dnmt1 expression levels affected all.

### Table I. Chi-square table for progeny generated from the triple cross

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<th>Total</th>
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<tr>
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<td>46 (47.6)</td>
<td>49 (47.6)</td>
</tr>
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<td>21 (23.8)</td>
<td>27 (23.8)</td>
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<tr>
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<td>37 (23.8)</td>
</tr>
<tr>
<td>Total</td>
<td>217 (190.4)</td>
<td>195 (190.4)</td>
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</table>

Values outside of parenthesis represent observed values. Values inside the parenthesis represent expected values. χ² = 12.078, d.f. = 15, P-value 0.67. Min, multiple intestinal neoplasia.

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**Fig. 1.** Histology and immunohistochemistry of pancreas sample from 100 days old Apc<sup>Min+</sup>, Trp53<sup>C0</sup>, Dnmt1<sup>R+</sup> mouse. Top-left panel represents hematoxylin and eosin (top) and β-catenin-stained (bottom) sections of normal pancreatic tissue (left) with an ×4 higher magnification of the boxed region (right) (A) Top-right panel represents hematoxylin and eosin (top) and β-catenin-stained (bottom) sections of focus where cluster of immature pancreatic cells can be seen (left) with an ×4 higher magnification of the boxed region (right) (B). Bottom-left panel represents hematoxylin and eosin (top) and β-catenin-stained (bottom) sections of adenoma where cluster of well-differentiated pancreatic cells with increased nuclear size can be seen (left) with an ×4 higher magnification of the boxed region (right) (C) Bottom-right panel represents hematoxylin and eosin (top) and β-catenin-stained (bottom) sections of carcinoma where large area of ill-defined boundary and loss of pancreatic cellular architecture can be seen (left) with an ×4 higher magnification of the boxed region (right) (D).
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We therefore analyzed the representation of each type of lesion with decreasing Dnmt1 levels. This analysis would clarify whether the diminishing trend of tumorigenesis is due to the decrease in number of low-grade lesions or high-grade lesions or both. Absolute counts of foci (Figure 2B), adenomas (Figure 2C) and carcinomas (Figure 2D) per mouse were plotted per β-catenin immunostaining. The number of lesions (atypical foci, adenomas and carcinomas) decreased significantly with diminishing Dnmt1 levels (Figure 2B–D). The results suggest that the decrease in overall tumor burden is independent of particular lesion type and that the decrease in Dnmt1 levels significantly affects the formation of foci, adenomas and carcinomas.

Reduction in Dnmt1 levels does not affect tumor size

We also examined whether reduced Dnmt1 levels has an effect on tumor size. We calculated the mean area of foci (Figure 3A) per mouse and mean area of adenoma (Figure 3B) per mouse. We found that Dnmt levels are not significantly associated with benign lesion size across the genotypes. We were not able to assess the effect of reduced levels of Dnmt1 on malignant lesions, due to their low numbers and poorly circumscribed borders.

Aberrant DNA methylation alterations is observed in several candidate loci

The reduced frequency of pancreatic tumors in Dnmt1 hypomorphic mice suggests that Dnmt1, or its resulting methylation, may contribute to tumorigenesis in the normal pancreas. Aberrant hypermethylation of certain genes, such as tumor suppressors, may predispose normal pancreatic cells to undergo oncogenic transformation. Therefore, we used MethyLight assay to determine whether alterations in promoter methylation is observed in the normal pancreas as well as tumor of Dnmt1 hypomorphic mice. Candidate genes from our collection were chosen based on literary knowledge of their methylation or expression status in tumors of mouse pancreatic cancer model and human pancreatic cancer. In addition, reactions targeting well-characterized tumor suppressor genes and repetitive elements were used to assess local and global DNA methylation status.

Histologically, normal pancreatic tissue from ApcMin+/+, Trp53+/−, Dnmt1+/+ females with ApcMin+/+, Trp53+/−, Dnmt1R/+ male to generate 24 possible genotypes in offspring (A). The graph represents tumor burden index of the four important genotypes where the horizontal lines represent the mean and the whiskers represent standard deviation (A). Poisson Regression was used to calculate the P-value for trend (P < 10−12) (A). Number of foci (B) adenoma (C) and carcinoma (D) are plotted where the x-axis represents the Dnmt1 hypomorphic alleles and the y-axis represents the number of lesions per mouse. The horizontal bars represent the mean and the error bars represent standard deviation. Poisson Regression was used to calculate the P-values for trend. P-value for focus multiplicity, P < 0.0001 (B), adenoma multiplicity (C), P < 0.001 and carcinoma multiplicity, P = 0.03 (D).

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Reduction of pancreatic acinar cell tumor multiplicity

Fig. 3. Mean area of atypical foci and adenomas. Mean area of foci per mouse (A) and mean area of adenoma per mouse (B) are represented in similar fashion. The x-axis represents hypomorphic alleles of Dnmt1. The y-axis represents the mean area of foci (A) and adenoma (B) for each mouse. The horizontal bars represent the median. Results not statistically significant.

Fig. 4. DNA methylation analysis. MethyLight analysis was performed on pancreatic samples from mice in the cohort. Non-tumor hematoxylin and eosin stained sections from Apc\(^{Min\text{/+}}\), Trp53\(^{-/-}\), Dnmt1\(^{+/+}\) (n = 7) (black bars), Apc\(^{Min\text{/+}}\), Trp53\(^{-/-}\), Dnmt1\(^{+/+}\) (n = 3) (dark-gray bars), Apc\(^{Min\text{/+}}\), Trp53\(^{-/-}\), Dnmt1\(^{+/0}\) (n = 4) (light-gray bars), Apc\(^{Min\text{/+}}\), Trp53\(^{-/-}\), Dnmt1\(^{0/0}\) (n = 6) (white bars) were microdissected and subjected to MethyLight analyses (A). Each bar represents the mean PMR within each genotypic category and is normalized to Apc\(^{Min\text{/+}}\), Trp53\(^{-/-}\), Dnmt1\(^{+/+}\) levels. Error bars represent standard error. Asterisk (*) indicates P-value < 0.05 (linear regression). MethyLight analysis was also performed on matched tumor/normal pairs (B). Parallel plots represent the methylation level of each sample per raw PMR calculations.

single-copy loci showed decrease in methylation levels when comparing histologically normal pancreas from Apc\(^{Min\text{/+}}\), Trp53\(^{-/-}\), Dnmt1\(^{+/+}\) mice to Apc\(^{Min\text{/+}}\), Trp53\(^{-/-}\), Dnmt1\(^{0/0}\). However, only four genes Igf2/DMR2 (P < 10\(^{-5}\)), Vav1 (P < 10\(^{-6}\)), Gaia3 (P < 0.01) and Opcml (P < 0.03) demonstrated statistical significance when the trend test was applied.

In Figure 4B, a schematic representation in the form of a parallel plot is used to compare the DNA methylation levels of the candidate loci in matched normal and tumor (carcinoma) pairs (n = 10). Only samples with large carcinomas were considered for the analysis to ensure that substantial amount of DNA could be isolated. The plot represents raw PMR values for each of the 10 normal and tumor pairs for each gene. In cases with overlapping measurements, only one line can be seen. Based on the P-values generated, there are significantly higher levels of DNA methylation in tumor compared with normal samples for Igf2/DMR2 (P < 0.02) and Opcml (P = 0.02 and P = 0.02, respectively). However, there is also significant decrease in DNA methylation levels in tumor compared with normal samples for Igf2/DMR2 (P < 0.02) and marginally significant decrease in DNA methylation of Vav1, B2, IAP (P = 0.06, 0.07 and 0.08, respectively). Together, the methylation data suggests that there are significant alterations in methylation levels of some loci in the Dnmt1 hypomorphic background compared with pancreatic tissue from wild-type Dnmt1 mice.

Discussion

Previous studies have demonstrated the importance of the major maintenance DNA methyltransferase Dnmt1 for tumor development (6–9,12–14). In this study, we have used a mouse model of exocrine pancreatic cancer to further study the effect of reduced levels of functional maintenance DNA methyltransferase Dnmt1. We observed a decrease in tumor burden throughout different stages of pancreatic tumor development with reduced levels of Dnmt1 expression. This suggests that Dnmt1 is important for the development of pancreatic neoplasia in mice.

Reduction of Dnmt1 levels has been shown to possess dual, yet opposing effects on tumorigenesis, suppression of advanced stage tumors (6–9,12,14) and promotion of early lesions and other types of tumors as a result of chromosomal instability (8,12,13,23). In our model, we categorized the lesions as atypical foci, considered to be early stage lesions, along with adenomas and carcinomas. In line with previous reports for other tumor model systems, we observed a significant reduction in the number of adenomas and carcinomas with decreasing Dnmt1 expression levels (6–9,12,14). This suggests that a decrease in Dnmt1 expression may suppress pancreatic tumor development by reducing the occurrence of epigenetic silencing of key tumor suppressor genes, as has been purposed in other studies of murine tumor models (14,24). The decrease in tumor burden could be explained by one of several mechanisms, including a decrease rate of tumor initiation, or a delayed onset of tumor initiation, and/or a decreased rate of tumor progression as a consequence of insufficient Dnmt1 expression. The fact that the reduction in tumor burden is observed across all stages suggests that both tumor initiation and the rate of tumor progression may be affected by the Dnmt1 hypomorphic alleles.

Others have previously reported an increase in microadenomas in the intestine and livers of Apc\(^{Min\text{/+}}\), Dnmt1-hypomorphic mice (12).
Surprisingly, we observed a decrease in early-stage pancreatic lesions, rather than an increase, suggesting that pancreatic early-stage lesions may not be as reliant on cytogenetic instability, as intestinal and hepatic early-stage lesions. However, β-catenin accumulation in the early-stage pancreatic lesions in our model suggests loss of heterozygosity of the Apc locus. One explanation for this apparent inconsistency could be the use of Dnmt1-hypomorphic mice by Yamada et al., which have lower levels of functional Dnmt1 expression, compared with Dnmt1+/+ mice (12,25,26). The degree of DNA hypomethylation in the Dnmt1−/− mouse may not be low enough to result in an increase in microadenomas or it may be that chromosomal instability caused by DNA hypomethylation may not be the prevailing mechanism for neoplastic proliferation in the early stages of tumorigenesis within this pancreatic model. The discrepancy may also reflect differences in the mouse strains used in the two studies. The strain background is an important factor affecting the susceptibility of ApcMin mice to tumor development (27).

The size of the atypical foci and adenomas of the pancreas was not altered in our tumor model. The areas of atypical foci were generally <0.03 mm² and adenomas were generally >0.03 mm² in area. Although we did not observe a significant difference in size of atypical foci and adenomas with decreasing Dnmt1 levels at 100 days of age, further aging of the mice may have revealed differences. Cormier et al. (9), reported that tumor growth rate in ApcMin/+ Dnmt1−/− mice compared with ApcMin−/− siblings diverge significantly after 70 days of age.

The importance of Dnmt1 expression has been studied in human pancreatic cancer tissues and cell lines (1–5,28,29). The significance of Dnmt1 protein expression was demonstrated in a multistage pancreatic carcinogenesis study (30). Peng et al. showed that Dnmt1 immunoreactivity rose significantly with increasing progression from peripheral pancreatic duct epithelia showing no remarkable histological findings without an inflammatory background to pancreatic intraepithelial neoplasia (PanIN). This finding also held true for different dysplastic grades of PanIN (from PanIN I to PanIN II) as well as PanIN to invasive carcinomas (30). A continuation of the previous study demonstrated increasing DNA methylation levels of a few key tumor-regulating genes (CDKN2A, APC, BRCA1 and TIMP3) with progressing histological grade, suggesting that DNA methylation is important in both early- and late-stage human pancreatic tumor formation (31).

In this study, we analyzed DNA methylation changes that accompany reduced tumor multiplicity in the presence of Dnmt1 hypomorphic alleles in ApcMin/+ Trp53−/− mouse model. We examined both global and gene-specific DNA methylation changes using three repetitive elements and 10 single-copy genes based on candidate gene approach. Hypermethylation of some genes, such as tumor suppressor genes, predisposes pre-neoplastic cells to undergo oncogenic transformation. It is probable that these aberrant changes in DNA methylation patterns can be observed in non-tumor tissue. Therefore, we performed DNA methylation analysis using normal pancreatic tissue to assess the percentage of methylated DNA molecules in the hypomorphic samples relative to wild-type. The analysis revealed a decreasing trend in methylation when comparing ApcMin−/−, Trp53−/−, Dnmt1−/− mice to ApcMin−/−, Trp53−/− with varying Dnmt1 hypomorphic combinations. A subset of the loci, such as GATA3, Vav1, Igf2/DMR2 and Opeml, showed a significantly decreasing trend with decreasing Dnmt1 expression. However, some of these loci (GATA3, IGFL2/DMR2 and VAV1) have been reported to be demethylated and aberrantly overexpressed in human pancreatic cancers, rather than hypermethylated and silenced (32–39). The discrepancy between these published observations in human tumors and our results could be attributable to differences in tumor type (insulinomas and ductal adenocarcinomas in humans and acinar cell carcinomas in our model).

Other genes show a DNA methylation behavior more consistent with tumor-associated gene silencing associated with promoter hypermethylation. Opeml has been reported to be hypermethylated in various cancers, most notably in the lung and ovary (40–46). In our study, Opeml showed a statistically significant decreasing trend in DNA methylation levels in the hypomorphic mice. Interestingly, we observed a significant increase in DNA methylation of the locus in tumors compared with match non-tumor adjacent tissues. Igf4 is also hypermethylated in tumor samples compared with non-tumor adjacent pancreatic tissue. ITGA4 has been described as a DNA methylation biomarker for the detection of colorectal cancer, bladder cancer and gastric cancer (47–50). We have previously reported hypermethylation of Igf4 in tumors from mouse models of intestinal cancers and subsequent loss of methylation with the introduction of the hypomorphic alleles (6,8).

We observed a reduction in DNA methylation levels for a few key tumor suppressor genes, such as Cdkn1a (p21) and Cdkn2a (p16) in normal pancreatic tissue from hypomorphic mice compared with mice with intact Dnmt1 levels, although results obtained in this study did not reach statistically significance, perhaps due to the limited sample size. This was also the case for Cdkn2a in the tumor versus non-tumor comparison (Figure 4B).

The DNA methylation levels of the repetitive elements in our study demonstrate comparable levels between Dnmt1 wild-type and the hypomorphic alleles. This is consistent with a previous observation that IAP repetitive elements are resistant to DNA methylation changes during epigenetic reprogramming of mouse primordial germ cells (51–53). This resistance to methylation changes of the IAP elements may be a protective measure against retrotransposition of the IAP. Previous work by Liang et al. clarifies the compensatory role of de novo Dnmts (Dnmt 3a/3b) for inefficient maintenance methylation by Dnmt1 of LINE-1 repetitive elements, which suggests co-operation between de novo and maintenance Dnmts in maintaining methylation of repetitive elements (54).

In humans, tumors of the pancreas present slightly different characteristics compared with the ApcMin−/−, Trp53−/− acinar model. The majority of human pancreatic cancers are of exocrine origin in the form of ductal adenocarcinomas. Both APC and TP53 mutations are observed in human pancreatic cancer. APC mutations are largely observed in acinar cell carcinoma, pancreatoblastoma and solid pseudopapillary neoplasm (55). On the other hand, TP53 genetic alterations are largely found in pancreatic ductal adenocarcinomas. Even though pancreatic ductal adenocarcinoma is the most common type of pancreatic cancer in humans, the cellular origin of the disease has been questioned. Although the cells bear strong similarities to ductal cells, some believe that the origin of these tumor cells is in fact acinar (56,57). Acinar cells make up the majority of the pancreas. Therefore, the use of a mouse model that develops pancreatic acinar cell carcinoma has allowed us to study the role of Dnmt in cells that have the highest representation in the pancreas.

Previous publications have demonstrated that 5-aza-2′-deoxycytidine treatment of pancreatic cancer cell lines inhibits cellular proliferation (28,29,58). The important finding of our research is that in vivo alterations of Dnmt levels have a significant effect on both early- and late-stage pancreatic tumor formation. This knowledge could potentially be used in a clinical setting to treat pancreatic cancer in both early and advanced stages of the disease using epigenetic therapeutic agents.

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Reduction of pancreatic acinar cell tumor multiplicity


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