(13)　研究結果の公表

ご協力によって得られた結果は、個人が誰であるか分からないようにした上で、学会や学術雑誌、データベース上などで公に発表されることがあります。

(14)　試料等の保存、使用及び廃棄の方法

提供いただいた膵臓組織は自治医科大学ゲノム機能研究部において厳重に保管し、本研究のために使用されます。しかし、あなたが同意してくだされば、将来の研究のための貴重な資源として研究終了後も保管させていただきます。この場合も、(9)で説明した方法により、誰の試料か分からないようにしたまま、試料を使い切るまで保管します。試料を廃棄する場合は、匿名のまま密封容器に廃棄するか又は焼却処分します。将来、試料を医学研究に用いる場合には、改めて研究計画書を提出し、自治医科大学生命倫理委員会等の承認を受けます。

(15)　遺伝カウンセリングの利用

病気のことや遺伝子解析に関して、不安に思ったり、相談したいことがある場合は、担当医にお申し付け下さい。研究責任者の間野博行がご相談に応じます。また必要に応じて自治医科大学附属病院遺伝カウンセリング室において専門のカウンセリングを受けることもできますが、この場合は一回目 6,000 円、二回目以降 4,300 円の自由診療料金をあなたに負担していただくことになります。

(16)　試料等の提供は無償・無報酬

遺伝子解析は研究費によって行なわれますので、あなたが費用を負担することはありません。また、この研究への協力に対して、あなたへの報酬は支払われません。

この研究の費用は、公的機関を中心とした科学研究費によっています。

(17)　問い合わせ、苦情の受付

この遺伝子解析研究についてのお問い合わせは、研究責任者までご連絡下さい。苦情がある場合は、自治医科大学大学事務部学事課(電話 0285-44-7044)で受け付けます。

平成　年　月　日

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（膵液採取用）

同意書

自治医科大学学長　高久　史麿　殿

私は膵疾患の病態解明目的の研究：課題名「ゲノミクス技術を用いた膵疾患の病態解析」について、（説明者氏名）＿＿＿＿＿＿から説明文書を用いて説明を受け、その方法、危険性、分析結果のお知らせの方法等について十分理解しました。ついては、次の条件で研究に協力することに同意します。

説明を受け理解した項目（□の中にご自分でチェックの印を付けてください。）

□ 遺伝子について
□ 研究の協力は任意で協力しなくても不利益を受けないこと。同意の撤回も文書によって自由にできること。
□ 研究の目的と方法
□ 希望により研究計画書を見ることができること。
□ 試料等提供者にもたらされる利益と不利益
□ 個人情報の保護の方法
□ 遺伝子解析結果の説明の方針
□ 研究結果の公表
□ 研究から財産権が生じても試料等提供者には帰属しないこと。
□ 研究終了後の試料等の取扱の方針
□ 解析に関する費用負担は無く、試料等の提供に対する報酬の支払いも無いこと。
□ 希望により遺伝カウンセリングが受けられること。

1　私は上記の項目のすべての□にチェックの印を記入した上で、私の提供する膵液試料が、本遺伝子解析研究に使用されることに同意します。

本人署名又は記名・捺印

２　上記１で同意された方は、下記の２－１又は２－２のどちらかを選択し、番号を丸で囲み、署名又は記名・捺印してください。

２－１　提供する試料等を本研究のみに使用し、かつ本研究の終了時には速やかに破棄してください。

２－２　提供する試料等が本研究に使用されるとともに長期間保存され、将来新たに計画・実施される遺伝子の解析を含む医学研究に使用されることに同意します。

本人署名又は記名・捺印

平成　　年　　月　　日

本人の氏名

住所

電話

説明者の職名・氏名

説明者の署名又は記名・捺印

（膵臓採取用）

同意書

自治医科大学学長　高久 史麿 殿

私は膵疾患の病態解明目的の研究：課題名「ゲノミクス技術を用いた膵疾患の病態解析」について、（説明者氏名）＿＿＿＿＿＿から説明文書を用いて説明を受け、その方法、危険性、分析結果のお知らせの方法等について十分理解しました。ついては、次の条件で研究に協力することに同意します。

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- □ 試料等提供者にもたらされる利益と不利益
- □ 個人情報の保護の方法
- □ 遺伝子解析結果の説明の方針
- □ 研究結果の公表
- □ 研究から財産権が生じても試料等提供者には帰属しないこと。
- □ 研究終了後の試料等の取扱の方針
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- □ 希望により遺伝カウンセリングが受けられること。

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本人署名又は記名・捺印

平成　　年　　月　　日

本人の氏名

住所

電話

説明者の職名・氏名

説明者の署名又は記名・捺印

厚生労働科学研究費補助金（化学物質リスク研究事業）
分担研究報告書
「化学物質の情報収集と選択」に関する研究

分担研究者　香山　不二雄　自治医科大学保健科学教授

研究要旨

化学物質を対象としたToxicogenomics研究に関する最新の論文をレビューし、その現状を把握した。その結果、Toxicogenomicsは腎毒性や肝毒性を惹起する化学物質の毒性発現の機序解明に多く用いられており、かなり成果が期待できるが、発癌性や変異原性などの解明にはその応用は限られていることが明らかになった。今後Toxicogenomicsを通常の毒性学的研究に利用するためには、さらに多くの情報を蓄積し、意志決定をするための評価システムを構築する必要がある。

A. 研究目的

化学物質を対象とした Toxicogenomics 研究に関する最新の情報を収集し、今後の研究に必要な情報を選択する。

B. 研究方法

Toxicogenomics 研究に関する最新の論文を検索し、その研究成績をまとめる。

C. 研究結果、D. 考　察

Toxicogenomics の研究では、いろいろな化学物質が検討されてきた。特に、腎毒性、肝毒性を惹起する化学物質の毒性発現機序に関する研究には、多くの物質が使用されている。

これまでに検討され近年報告されている化学物質は cadmium (Tan Y, 2006) Indole-3-Carbinol, 17[beta]-Estradiol and [beta]-Naphthoflavone (Tilton SC, 2006)、Polycyclic aromatic hydrocarbons (PAHs) (Staal YCN et al. 2006) Fumonisin mycotoxins (Voss et al. 2006)などがある。

ochratoxin A は食品に発生するカビ毒 (mycotoxin) の一種であり、肝臓毒性、腎毒性が顕著で、腎癌を誘導する。DNA microarray 解析では、細胞障害および組織再生に関与する遺伝子が誘導される。特に、DNA 障害と DNA 修復に関与する遺伝子群、カルシウム平衡に関する遺伝子、転写因子の transcription factors hepatocyte nuclear factor 4 alpha (HNF4alpha) と nuclear factor-erythroid 2-related factor 2 (Nrf2) が誘導されるが、この現象は腎臓だけで肝臓では見られない。以前の報告から考察すると HNF4alpha が腎臓の発癌に関与していると考えられる。Nrf2 により制御されている遺伝子が解毒や抗酸化ストレスに対応していること明らかになっている。これらの遺伝子を欠損や抑制をすると、細胞毒性をより顕著にすることが予想される(Marin-Kuan, 2006)。

しかし、網羅的な発現誘導および抑制された遺伝子群の結果をどのよう使用するかが重要である。クラスター解析などが試みられてい

るが、より生理学や代謝、薬物動態学に基づいた解析の主体が移りつつある。

Toxicogenomics の実際に研究する場合、細胞株を使う場合と初代培養細胞を使用する場合では、得られる結果が異なり、評価の仕方も異なることを注意する必要がある。HepG2 細胞株とヒト肝細胞初代培養細胞を用いた結果では、HepG2 細胞では 30%の反応する遺伝子が失われており、その意味は限定的である。また、初代培養細胞ではドナーによる違いがあり、その薬物代謝や反応性に個人差を内在すること、すなわち関連する遺伝子群の SNPs などの差異を含んでいることを考慮する必要がある。しかし、この解析から個人の化学物質および薬物に対する反応性の遺伝的多様性につながっていく(Harris et al. 2004)。

医薬品や毒物に対する個人の感受性の違いについて、近年、toxicogenomics および pharmacogenomics の知見から、P450 酵素群および TMTP に代表されるような phase2 酵素群などの SNPs の違いによるもので説明されるようになっている。細胞内取り込みに関与する膜タンパク質、レセプター、シグナル伝達に関与するタンパク群、薬物代謝酵素などの遺伝子多型が関与していることが明らかになってきている。最近、phase I 酵素群、cytochrome P450 1A1, 1A2, 1B1, 2C9, 2C19, 2D6, 3A5 、myeloperoxidase、phase II 酵素群、 arylamine N-acetyltransferases 1 と 2、 glutathione S-transferases M1 と T1, と thiopurine S-methyltranferases などの SNPs との関与が明らかとなっている(Cascorbi, 2005, Oberemm et al., 2005)。

Toxicogenomics の応用として、毒物の作用機序の研究、量-反応関係に関する研究などでは、かなり成果が期待できる。発癌性、変異原性などの解析について現在まだ利用可能性は限定的であるが、新しい化学物質の作用機序に関する情報は toxicogenomics の中でも transcriptomics の情報が役に立ち、多くの通常の毒性学的実験研究や解析の労力を節約することができる可能性がある。もちろん、そのためにはさらに多くの情報を蓄積収集し、意志決定をするための評価システムを構築していく必要がある(Battershill, 2005)。

Transcriptomics, proteomics および metabolomics は toxicogenomics の中核的技法であり、これまでの通常の毒性学における遺伝子、タンパク質、代謝物の発現系を統合して評価する手法となる可能性がある。まず、toxicogenomicsh はその sensitivity の高さに期待が集まった。しかしそれ以上に、toxicogenomics により得られる知見は、毒性発現機序のよりよい理解をもたらすと同時に、その新たな利用法は、むしろ未知の化学物質の毒性を評価することを容易にする可能性がある。化学物質の有害影響を見いだすのに有効に働く可能性もある。特に、これまで大変難しかった複数の化学物質の混合物の評価に向いており、化学物質の in vivo での新しい作用機序やその経路についての新たな知見を実際にもたらしつつある。(Stierum R et al., 2005)

さらに、pharmacogenomics および toxicogenomics の実用的利用として、腎移植後の免疫抑制剤の投与量設定などがある。すなわち、免疫抑制剤の投与量が少なすぎて免疫抑制不全または多すぎて毒性発現する。そのため、適切な投与量や組み合わせを in vitro と in vivo で繰り返すことができる。このように、いろいろな応用のフィールドは今後発展していくと考えられる(Thukral et al. 2005)。

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E. 結　論

今後 Toxicogenomics を通常の毒性学的研究に利用するためには、さらに多くの情報を蓄積し、意志決定をするための評価システムを構築する必要がある。

F．健康危険情報

なし

G．研究発表

1) 国内

口頭発表	0 件
原著論文による発表	0 件
それ以外(レビュー等)の発表	0 件

発表論文

学会発表

2) 海外

口頭発表	0 件
原著論文による発表	0 件
それ以外(レビュー等)の発表	0 件

発表論文

学会発表

H．知的財産権の出願・登録状況

1. 特許取得；なし
2. 実用新案登録；なし
3. その他；なし

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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健康危険情報

なし

Oncogene (2006) 25, 139–146

www.nature.com/onc

SHORT COMMUNICATION

Epigenetic silencing of *AXIN2* in colorectal carcinoma with microsatellite instability

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Mutation or epigenetic silencing of mismatch repair genes, such as *MLH1* and *MSH2*, results in microsatellite instability (MSI) in the genome of a subset of colorectal carcinomas (CRCs). However, little is yet known of genes that directly contribute to tumor formation in such cancers. To characterize MSI-dependent changes in gene expression, we have now compared transcriptomes between fresh CRC specimens positive or negative for MSI ($n = 10$ for each) with the use of high-density oligonucleotide microarrays harboring >44 000 probe sets. Correspondence analysis of the expression patterns of isolated MSI-associated genes revealed that the transcriptome of MSI$^+$ CRCs is clearly distinct from that of MSI$^-$ CRCs. Such MSI-associated genes included that for AXIN2, an important component of the WNT signaling pathway. *AXIN2* was silenced, apparently as a result of extensive methylation of its promoter region, specifically in MSI$^+$ CRC specimens. Forced expression of *AXIN2*, either by treatment with 5′-azacytidine or by transfection with *AXIN2* cDNA, resulted in rapid cell death in an MSI$^+$ CRC cell line. These data indicate that epigenetic silencing of *AXIN2* is specifically associated with carcinogenesis in MSI$^+$ CRCs.

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Colorectal carcinoma (CRC) is one of the leading causes of cancer death in humans. Evidence indicates the existence of two major types of genomic instability in CRCs: chromosomal instability and microsatellite instability (MSI) (Lengauer *et al.*, 1998). Whereas chromosomal instability is associated with an abnormal DNA content (such as aneuploidy), inactivation of the tumor suppressor gene *TP53*, and activation of oncogenes (Kinzler and Vogelstein, 1996), MSI is associated with defects in DNA mismatch repair (MMR) that result in frameshift mutations in microsatellite repeats and thereby affect the structure of genes containing such repeats (Ionov *et al.*, 1993).

Although germline mutations of MMR genes have been detected in the genome of individuals with hereditary nonpolyposis colorectal cancer (Fishel *et al.*, 1993; Bronner *et al.*, 1994; Papadopoulos *et al.*, 1994), many sporadic CRCs positive for MSI are associated with epigenetic silencing of nonmutated MMR genes (Toyota *et al.*, 1999; Miyakura *et al.*, 2001). MSI$^+$ CRCs are characterized by specific clinicopathologic features and gene mutations. They occur with a higher frequency in women than in men, develop in the right side of the colon, and manifest a mucinous or poorly differentiated histopathology. Many of the CpG dinucleotides within the promoter region of the MMR gene *MLH1* are methylated (Cunningham *et al.*, 1998; Veigl *et al.*, 1998) and the *BRAF* gene frequently contains activating mutations (Koinuma *et al.*, 2004) in MSI$^+$ CRCs. Multiple genomic fragments have been found to be methylated in such CRCs (Toyota *et al.*, 1999), and an entity of CRC with a CpG island methylator phenotype has been proposed (Issa, 2004). The repertoire of genes that become methylated specifically in CRCs positive for *MLH1* methylation has remained uncharacterized, however.

To characterize directly the transcriptome specifically associated with MSI$^+$ CRC, we have now compared transcriptomes between fresh CRC specimens with or without MSI. Unexpectedly, we found that the expression of *AXIN2*, which encodes a component of the WNT signaling pathway, was markedly suppressed among the former tumors. CpG sequences within the *AXIN2* promoter were revealed to be extensively methylated in such CRCs. Forced expression of *AXIN2* inhibited cell proliferation in an MSI$^+$ CRC cell line, indicating that loss of *AXIN2* transcription is directly associated with carcinogenesis in MSI$^+$ CRCs.

To identify genes whose expression is specifically altered in MSI$^+$ CRCs, we first compared the transcriptomes of CRCs with or without MSI. A total of 248 consecutive cases of CRC were examined for MSI status

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as well as for methylation of the promoter region of *MLH1* (Koinuma *et al.*, 2004). Most ($n=213$) of the cancer specimens were MSI$^-$, with the remainder ($n=35$) being positive for MSI. To compare the transcriptomes of these two subtypes of CRC, we randomly selected 10 specimens from each group and subjected them to gene expression profiling with microarrays (Affymetrix GeneChip HGU133) that harbor >44 000 probe sets. The clinical characteristics of the patients whose CRC specimens were subjected to microarray analysis are summarized in Table 1.

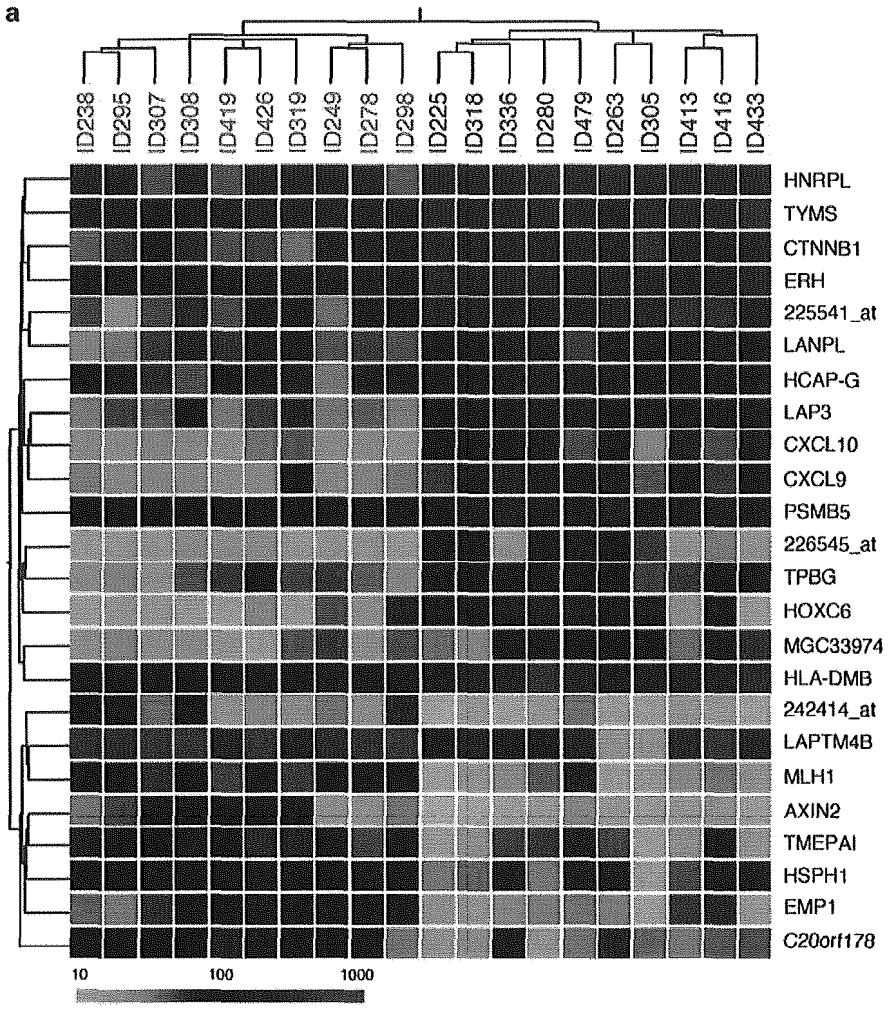
To exclude transcriptionally silent genes from our analyses, we first chose probe sets that received the 'Present' call from Microarray Suite 5.0 (Affymetrix) in at least 10% ($n=2$) of the samples. Two-way hierarchical clustering (Alon *et al.*, 1999) of the 20 patients based on the expression profiles of the isolated 21 888 probe sets failed to separate those with MSI$^+$ CRC from those with MSI$^-$ CRC (data not shown). We therefore attempted to identify 'MSI-associated probe sets' whose expression intensities differed significantly (Student's t-test, $P<0.001$) between the two classes and whose effect size (absolute difference in mean expression level) was $\geqslant 50$ U. Two-way clustering analysis with the 24 probe sets that fulfilled both these criteria clearly separated the individuals of the two clinical classes (Figure 1a). The distinct transcriptomes of the two classes were also confirmed by correspondence analysis (Fellenberg *et al.*, 2001), which reduced the complexity of the gene expression patterns from 24 to three dimensions. Projection of the study subjects into a virtual three-dimensional space based on their calculated coordinates revealed that the MSI$^+$ specimens were positioned apart from the MSI$^-$ ones (Figure 1b). These data indicate that the two classes of CRC possess distinct gene expression profiles, or 'molecular signatures', and they also suggest the feasibility of gene expression-based differential diagnosis of the two CRC subtypes.

Table 1 Clinical characteristics of the study subjects enrolled in microarray analysis

Patient ID	*Age (years)*	*Sex*	*MSI status*	MLH1 *methylation*	BRAF *gene*	KRAS2 *gene*	*Tumor site*	*Dukes stage*	*Pathology*	AXIN2 *methylation*
225	83	Female	Positive	Yes	Mutant	Wild	Proximal	C	Well	Yes
263	86	Female	Positive	Yes	Mutant	Wild	Proximal	C	Mod	Yes
280	83	Female	Positive	Yes	Mutant	Wild	Proximal	C	Well	Yes
305	74	Male	Positive	Yes	Mutant	Wild	Proximal	B	Sig	No
318	76	Female	Positive	Yes	Mutant	Wild	Proximal	B	Well	Yes
336	68	Male	Positive	Yes	Mutant	Wild	Proximal	B	Muc	No
413	69	Female	Positive	Yes	Mutant	Wild	Proximal	A	Well	No
416	76	Female	Positive	Yes	Mutant	Wild	Proximal	B	Muc	No
433	54	Female	Positive	Yes	Wild	Wild	Proximal	D	Well	Yes
479	74	Female	Positive	Yes	Mutant	Wild	Proximal	B	Mod	No
238	74	Male	Negative	No	Wild	Mutant	Distal	A	Well	No
249	62	Male	Negative	No	Wild	Wild	Proximal	B	Well	No
278	73	Male	Negative	No	Wild	Wild	Proximal	C	Well	No
295	71	Female	Negative	No	Wild	Mutant	Proximal	C	Well	No
298	70	Male	Negative	No	Wild	Mutant	Proximal	D	Well	No
307	80	Female	Negative	No	Wild	Wild	Proximal	C	Mod	No
308	62	Male	Negative	No	Wild	Wild	Distal	B	Mod	No
319	53	Female	Negative	No	Wild	Wild	Distal	A	Well	No
419	45	Female	Negative	No	Wild	Mutant	Proximal	D	Muc	No
426	42	Female	Negative	No	Wild	Wild	Proximal	C	Well	No

Well = well-differentiated adenocarcinoma; Mod = moderately differentiated adenocarcinoma; Sig = signet ring cell adenocarcinoma; Muc = mucinous adenocarcinoma. Methylation of *AXIN2* promoter region was determined by COBRA method.

Figure 1 Comparison of transcriptomes between CRCs positive or negative for MSI. (**a**) Subject tree generated by two-way clustering analysis with 24 probe sets that contrasted the two clinical conditions ($P<0.001$; effect size, $\geqslant 50$ U). Tumor samples were obtained from individuals with sporadic CRC who underwent surgical treatment at Jichi Medical School Hospital. Written informed consent was obtained from all patients, and the present study was approved by the ethics committee of Jichi Medical School. Microsatellite stability was determined by analysis of nine microsatellite repeat loci (three dinucleotide repeats and six mononucleotide repeats) as described previously (Miyakura *et al.*, 2001), and MSI status was stratified according to the criteria of the National Cancer Institute workshop (Boland *et al.*, 1998). Total RNA was extracted from ~100 mg of tissue, and was used in the hybridization experiments with GeneChip HGU133 A&B microarrays (Affymetrix), which harbor >44 000 probe sets corresponding to ~33 000 human genes, as described previously (Ohki-Kaneda *et al.*, 2004). The mean expression intensity of the internal positive control probe sets (http://www.affymetrix.com/support/technical/mask_files.affx) on the microarrays was set to 500 units (U) in each hybridization, and the fluorescence intensity of each probe set was normalized accordingly. All normalized array data are available at the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo) under the Accession Number GSE2138. Each column corresponds to a separate sample (MSI$^-$, green; MSI$^+$, red), and each row to a probe set whose expression is color-coded according to the indicated scale. Gene symbols are shown on the right; 225541_at, 226545_at, and 242414_at are expressed sequence tag IDs designated by Affymetrix (http://www.affymetrix.com). Annotations and expression intensities for the probe sets are presented in Supplementary Table 1. Note that *MLH1* expression was specifically suppressed in the MSI$^+$ samples. (**b**) Samples were projected into a virtual space with coordinates calculated by correspondence analysis of the 24 probe sets shown in (**a**). Correspondence analysis was performed with ViSta software (http://www.visualstats.org) for all genes showing a significant difference.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LAPTM4B
MLH1
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TMEPAI
HSPH1
EMP1
C20orf178
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1st dimension
MSI-positive
MSI-negative
0.4
0.2
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-0.2
-0.4
2nd dimension
-0.3
-0.2
-0.1
0.0
0.1
0.2
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3rd dimension

The isolated MSI-associated genes include *AXIN2* and *CTNNB1* (β-catenin), both of which encode key participants in the WNT signaling pathway (Tolwinski and Wieschaus, 2004). Dysregulation of ubiquitin-dependent degradation of β-catenin contributes to carcinogenesis in a variety of CRCs and hepatocellular carcinomas (Narayan and Roy, 2003). AXIN2, similar to AXIN1, functions as a scaffold protein to facilitate this ubiquitination process by recruiting adenomatous polyposis coli (APC), glycogen synthase kinase-3β, and β-catenin (Behrens *et al.*, 1998). Defects in the degradation of β-catenin have been shown to result from mutations in *AXIN1*, *AXIN2*, *APC*, or *CTNNB1* (Rubinfeld *et al.*, 1997; Liu *et al.*, 2000; Satoh *et al.*, 2000; Smith *et al.*, 2002). Our data therefore suggest that transcriptional suppression of *AXIN2* might represent a novel mechanism by which the function of the APC-AXIN–β-catenin complex is impaired in CRC.

To confirm the MSI-associated change in *AXIN2* expression, we measured the abundance of the corresponding mRNA in the original 20 study specimens by quantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis (Figure 2a). Comparison of the amount of *AXIN2* mRNA determined by RT–PCR with that determined by microarray analysis yielded a Pearson's correlation coefficient (r) of 0.89, indicating that the two data sets were highly correlated ($P<0.001$). (Also see Supplementary Figure 1 for verification of microarray data by RT–PCR.)

With the use of RT–PCR, we then measured the amount of *AXIN2* mRNA in a larger number of samples (seven additional specimens of MSI$^+$ CRC, for a total of 17; 10 additional specimens of MSI$^-$ CRC, for a total of 20; three MSI$^+$ CRC cell lines; two MSI$^-$ CRC cell lines). The abundance of *AXIN2* transcripts in most of the MSI$^+$ CRC specimens and cell lines was reduced compared with that in the MSI$^-$ ones (Figure 2b); an *AXIN2*/*ACTB* transcript ratio of $<5\times10^{-4}$ was apparent in 13 of the 17 MSI$^+$ CRC specimens, but in only five of the 20 MSI$^-$ ones (Fisher's exact probability test, $P=0.003$). Importantly, a similar MSI-dependent suppression of *AXIN1* expression was not observed among these specimens ($P=0.31$) (data not shown).

Human *AXIN2* possesses a relatively large CpG island within its promoter region (nucleotide positions, chr17: 60986365–60987824). We therefore examined the methylation status of the CpG sites within this region by nucleotide sequencing after sodium bisulfite treatment. Extensive methylation of the CpG island in the *AXIN2* promoter was apparent in CRC specimens positive for MSI and for the loss of *AXIN2* expression (Figure 2c). The promoter region in the MSI$^+$ CRC cell line HCT116 (Wheeler *et al.*, 1999) was also heavily methylated. The *MLH1* promoter in HCT116 cells is not methylated, but the coding sequence of the gene contains a mutation that results in MSI (Wheeler *et al.*, 1999).

On the basis of these findings, we examined the methylation status of the *AXIN2* promoter in 37 clinical specimens and five cell lines by combined bisulfite restriction analysis (COBRA) (Xiong and Laird, 1997). CpG methylation was detected in five of the 17 MSI$^+$ specimens, but in none of the 20 MSI$^-$ specimens (Table 1; see Supplementary Table 2). Methylation of the *AXIN2* promoter was not detected in normal colon tissue obtained from the individuals with MSI$^+$ CRC (data not shown), suggesting that *AXIN2* methylation was a somatic event in these patients.

We then tested whether the amount of the encoded protein correlated with that of *AXIN2* mRNA in CRC specimens (Figure 2d). Immunohistochemical staining showed that AXIN2 was abundant in a specimen with a high mRNA content (ID308), but was present in much smaller amounts in two specimens with a low mRNA content (ID263, ID295). Although a large amount of *AXIN2* mRNA was not always associated with a large amount of protein, a small amount of mRNA was consistently associated with a small amount of protein (data not shown).

To examine directly whether epigenetic silencing of *AXIN2* is relevant to the change in the growth properties of CRC cells, we restored *AXIN2* expression, either by 5′-azacytidine treatment or by introduction of *AXIN2* cDNA, in an MSI$^+$ CRC cell line. 5′-Azacytidine inhibits *de novo* methylation of genomic DNA and thereby induces demethylation of the genome of proliferating cells (Christman, 2002). HCT116 cells were incubated for 3 days with various concentrations of 5′-azacytidine and were then subjected to COBRA for determination of the methylation status of the *AXIN2* promoter. Treatment with 5′-azacytidine reduced the level of methylation of the *AXIN2* promoter in a concentration-dependent manner (Figure 3a). This effect of 5′-azacytidine was accompanied by an increase in the amount of *AXIN2* mRNA in the cells (Figure 3b) as well as by the induction of cell death (Figure 3c).

Given that 5′-azacytidine likely affects the transcription of other genes in addition to that of *AXIN2*, the growth inhibitory effect observed in HCT116 cells might not have been attributable solely to the induction of *AXIN2* expression. To examine the direct effect of *AXIN2*, we introduced its cDNA into HCT116 cells by transfection. However, an introduction of *AXIN2* cDNA (even with the use of an inducible system) resulted in rapid cell death, and we could not establish stable transformants of cell lines with such expression constructs (data not shown). Therefore, we generated an amphotropic recombinant retrovirus that confers simultaneous expression of both an MYC epitope-tagged form of AXIN2 and mouse CD8. Human kidney 293 cells infected with this virus, but not those infected with a mock virus, expressed AXIN2 (Figure 3d). HCT116 cells were then infected with the virus and were subjected to affinity chromatography 48 h thereafter to isolate cells that express CD8. Given that CD8-expressing cells would be expected also to express AXIN2, this column purification step should result in rapid enrichment of AXIN2-expressing cells. The isolated cells indeed contained a substantial amount of *AXIN2* mRNA as revealed by RT–PCR (Figure 3e). The purified CD8$^+$ HCT116 cells were then cultured for 3 days to characterize their growth properties. Forced expression of AXIN2 resulted in marked inhibition of cell growth

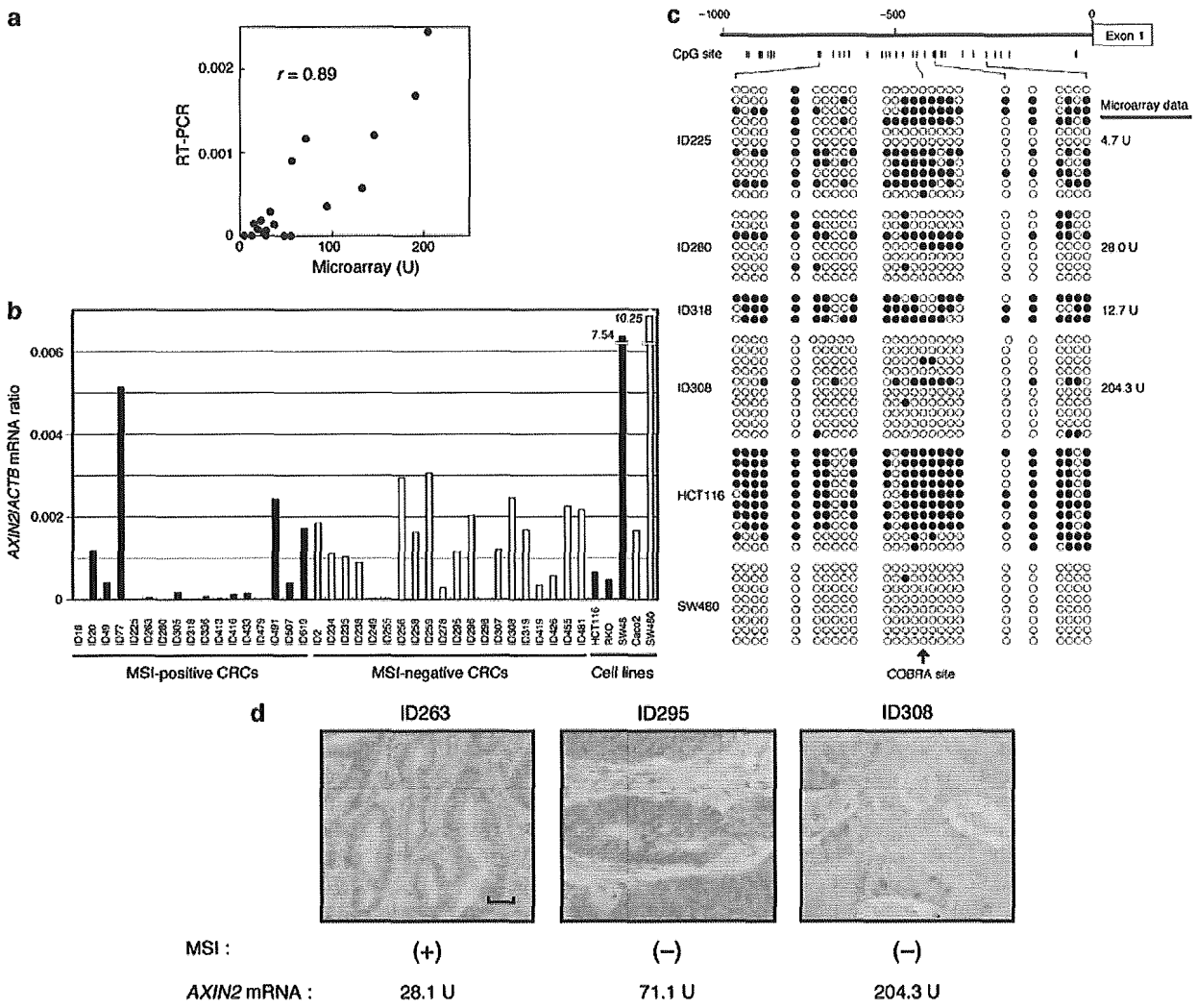


Figure 2 Suppression of *AXIN2* expression in CRCs positive for MSI. (**a**) Comparison of the abundance of *AXIN2* mRNA in study specimens as determined by microarray and RT–PCR analyses. For the latter, the amount of *AXIN2* mRNA was expressed relative to that of *ACTB* mRNA. Pearson's correlation coefficient (*r*) for the comparison is indicated. Portions of double-stranded cDNA were subjected to PCR with a QuantiTect SYBR Green PCR Kit (Qiagen). The amplification protocol comprised incubations at 94°C for 15 s, 63°C for 30 s, and 72°C for 60 s. Incorporation of the SYBR Green dye into PCR products was monitored in real time with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems), thereby allowing determination of the threshold cycle (C_T) at which exponential amplification of products begins. The amount of target cDNAs relative to that of the β-actin (*ACTB*) cDNA was calculated from the C_T values with the use of Sequence Detector ver. 1.6.3 software (PE Applied Biosystems). The primers used for PCR amplification were 5′-CTGGCTCCAGAAGATCACAAAG-3′ and 5′-ATCTCCTCAAACACCGCTCCA-3′ for *AXIN2* and 5′-CCATCATGAAGTGTGACGTGG-3′ and 5′-GTCCGCCTAGAAGCATTTGCG-3′ for *ACTB*. (**b**) Comparison of the amount of *AXIN2* mRNA relative to that of *ACTB* mRNA (as determined by RT–PCR) between MSI^+ (closed bars) and MSI^- (open bars) CRC specimens and cell lines. (**c**) Genomic DNA of the indicated clinical specimens and CRC cell lines was treated with sodium bisulfite (Koinuma *et al*., 2004), after which the *AXIN2* promoter region was amplified by PCR with the primers 5′-TTGTATATAGTTTA GYGGTTGGG-3′ and 5′-AAATCTAAACTCCCTACACACTT-3′. Closed and open circles indicate methylated and unmethylated CpG sites, respectively. The positions of the CpG sites are indicated at the top, the *Hha*I digestion site for COBRA is indicated by the arrow, and the microarray data for *AXIN2* expression are shown on the right. (**d**) Immunohistochemical analysis of the indicated clinical specimens with antibodies to AXIN2. The MSI status and the expression level of *AXIN2* determined by microarray analysis are indicated. Immunohistochemical analysis of AXIN2 expression was performed as described previously (Leung *et al*., 2002). Sections (5 μm) of formalin-fixed, paraffin-embedded tissue were mounted on Probe-On slides (Fisher Scientific), which were then incubated first for 1 h at room temperature with 1.5% normal horse serum and then overnight at 4°C with goat polyclonal antibodies to AXIN2 (Santa Cruz Biotechnology). Immune complexes were detected by the avidin–biotin–peroxidase method with 3,3′-diaminobenzidine as the chromogenic substrate (Vectastain ABC kit, Vector Laboratories). The sections were counterstained with hematoxylin. Scale bar, 50 μm.

(Figure 3f), indicating that silencing of *AXIN2* is indeed relevant to tumorigenesis. We also examined if the expression of *AXIN2* directly suppresses the WNT signaling pathway. For this purpose, we utilized a luciferase-based reporter plasmid (TOPflash) for the T-cell factor (TCF) activity, which is a direct target of

β-catenin (Korinek *et al.*, 1997). As shown in Figure 3g, a forced expression of *AXIN2* induced a marked suppression in the luciferase activity in HCT116 cells. On the other hand, *AXIN2* did not affect luciferase activity driven by a mutated, nonfunctional TCF-binding sites (FOPflash). These data clearly indicate that AXIN2 is involved in the WNT–APC–β-catenin pathway in CRCs.

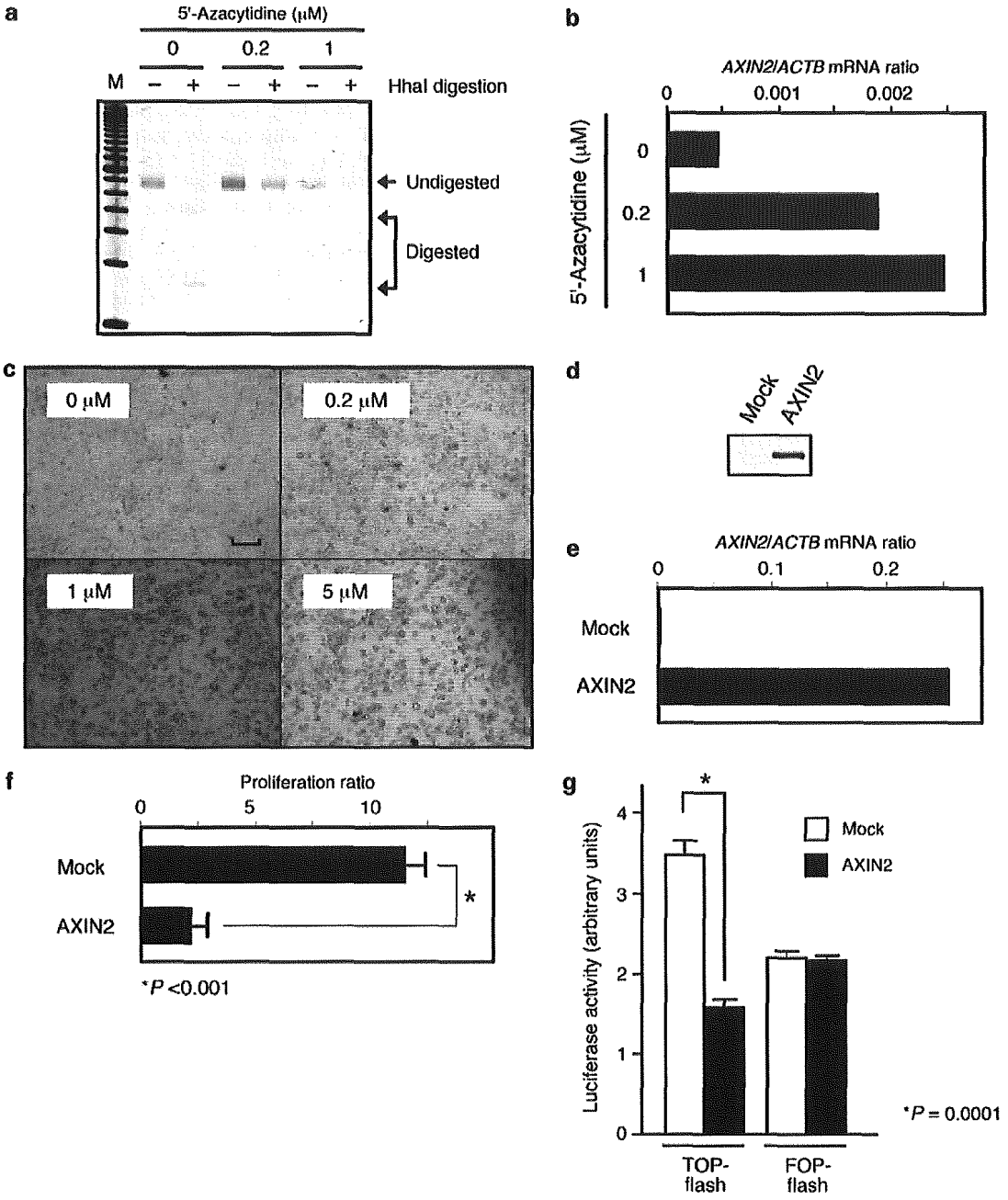
We have demonstrated preferential transcriptional silencing of *AXIN2* in MSI$^+$ CRCs. Recently, mutations within exon 7 of the *AXIN2* gene have been reported in MSI$^+$ CRC specimens (Liu *et al.*, 2000; Wu *et al.*, 2001). We have thus analysed the nucleotide sequence of the *AXIN2* gene among our MSI$^+$ samples ($n=9$). Sequencing of the *AXIN2* exon 7 has revealed that only one patient (ID no. 263) carried a mutated *AXIN2* gene in one allele (data not shown). A deletion of a cytosine residue at the nucleotide position 2096 of the *AXIN2* cDNA (GenBank Accession Number, AF078165) led to a frame shift in the open-reading frame in this patient, introducing a premature termination codon in AXIN2 protein at the amino-acid position of 688. However, majority of the patients had intact *AXIN2* genes, indicating that silencing, but not mutation, of *AXIN2* is the main pathway to impede the *AXIN2* function.

The COBRA experiments revealed that the promoter region of *AXIN2* was extensively methylated in MSI$^+$ CRCs but not in MSI$^-$ CRCs. Although the difference in the frequency of *AXIN2* methylation between these two classes of tumor was significant (Fisher's exact probability test, $P=0.003$), the frequency for the MSI$^+$ specimens was still only 29% and therefore was not able to account for all the observed instances of suppression of *AXIN2* expression. We judged COBRA data as positive for methylation if $\geqslant 10\%$ of the PCR products were digested by *Hha*I. However, a small proportion ($<10\%$) of the PCR products was digested in the analysis of $\sim 50\%$ of MSI$^+$ CRC specimens (data not shown), indicating that alterations in the methylation status of the *AXIN2* promoter were more widespread. It is therefore possible that CpG sites other than that targeted by COBRA are more frequently methylated in MSI$^+$ CRCs and are more important for transcriptional regulation.

Similar promoter methylation has been recently described for other genes important for the WNT signaling pathway. The genes for secreted frizzled-related proteins are thus epigenetically silenced in MSI$^+$ CRCs, resulting in constitutive activation of the WNT pathway (Suzuki *et al.*, 2004). CpG sites within the *APC* promoter were also found to be frequently methylated in CRCs and other cancers (Esteller *et al.*, 2000; Zysman *et al.*, 2002). These data thus suggest that not only genetic mutations but also epigenetic silencing might play an important role in tumorigenesis mediated by activation of the WNT pathway.

Methylation of the *APC* promoter in endometrial cancer has been shown to occur preferentially in MSI$^+$ tumors (Zysman *et al.*, 2002). Despite the lack of an MSI-associated difference in the expression of *APC* in our CRC specimens (data not shown), the results of this previous study together with our present findings suggest the possibility that genes related to the WNT signaling pathway are targeted for methylation specifically in cancers with MSI. Our data further indicate that such methylation in MSI$^+$ cancers may be directly relevant to the mechanism of malignant transformation through epigenetic silencing of tumor suppressor genes. MSI$^+$ CRCs have been thought to arise through genetic events distinct from those that underlie MSI$^-$ cancers (Rajagopalan and Lengauer, 2004), which are frequently associated with aneuploidy and mutations in WNT pathway genes such as *APC* and *CTNNB1*. However, our data indicate that the molecular mechanisms for malignant transformation overlap between MSI$^+$ and MSI$^-$ CRCs.

Figure 3 Induction of cell death by restoration of *AXIN2* expression in a CRC cell line with a methylated *AXIN2* promoter. (**a**) HCT116 cells were incubated for 72 h with 0, 0.2, or 1 μM 5′-azacytidine and were then subjected to COBRA for determination of the methylation status of the *AXIN2* promoter (Xiong and Laird, 1997). Genomic DNA was denatured, incubated for 16 h at 55°C in 3.1 M sodium bisulfite, and then subjected to PCR with the primers in Figure 2c. The PCR products were then digested with the restriction endonuclease *Hha*I (Takara Bio), and the resulting DNA fragments were fractionated by polyacrylamide gel electrophoresis. The gel was stained with SYBR Green I (Takara Bio) and scanned with an LAS3000 imaging system (Fuji Film). Genomic fragments were determined to be positive for CpG methylation if $\geqslant 10\%$ of the PCR products were cleaved by the restriction endonuclease. Lane M, DNA size markers (50-bp ladder). (**b**) The cells from (**a**) were also subjected to RT–PCR analysis for determination of the amount of *AXIN2* mRNA relative to that of *ACTB* mRNA. (**c**) Cells treated as in (**a**) with 0, 0.2, 1, or 5 μM 5′-azacytidine were examined by light microscopy. Cell death was estimated by counting the remaining viable cells in each culture dish by the dye-exclusion method. Scale bar, 50 μm. (**d**) Human kidney 293 cells infected with either a mock virus or a recombinant virus encoding both MYC epitope-tagged AXIN2 and mouse CD8. A human cDNA for AXIN2 tagged at its NH_2-terminus with the MYC epitope sequence was ligated into the pMX-iresCD8 retroviral plasmid (Yamashita *et al.*, 2001) to yield pMX-AXIN2-MYC-iresCD8. The latter plasmid was introduced into BOSC23 cells together with pE-ampho and pGP packaging plasmids (Takara Bio) by transfection with the use of Lipofectamine (Invitrogen). The culture supernatant containing recombinant viruses was added to 293 cells with 4 μg/ml of polybrene (Sigma). Cells were then subjected to immunoprecipitation with the antibodies to MYC (9E10, Roche Diagnostics), and to immunoblot analysis with the same antibodies. (**e**) HCT116 cells infected with the viruses in (**d**) were subjected to affinity chromatography to isolate CD8$^+$ cells, which were then subjected to RT–PCR analysis for quantitation of *AXIN2* mRNA relative to the amount of *ACTB* mRNA. (**f**) The CD8$^+$ fractions in (**e**) were seeded at a density of 5×10^4 cells/dish and cultured for 72 h, after which the ratio of the final cell number to the initial value was determined. Data are means + s.d. of triplicate from a representative experiment. The *P*-value for the indicated comparison was determined by Student's *t* test. (**g**) HCT116 cells were seeded at a density of 2.5×10^6 cells/6 cm dish. After 24 h of incubation, the cells were transfected, with the use of Lipofectamine, with 2 μg of pMX-AXIN2-MYC-iresCD8 (AXIN2) or pMX-iresCD8 (Mock). For the reporter plasmids, 0.5 μg of pGL4 (Promega, Madison, WI, USA) plus either 0.5 μg of pTOPflash or 0.5 μg of pFOPflash (both from Upstate Biotechnology, Lake Placid, NY, USA) were added to the lipofection mix. The activity of *Photinus pyralis* luciferase was measured after 24 h of incubation with the use of the Dual-luciferase reporter assay system (Promega), and normalized on the basis of the activity of *Renilla reniformis* luciferase produced by pGL4. Data are shown as the mean value + s.d. of triplicate samples.



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