To clarify the association between miRNA expression and survival in metastatic CRC patients, we limited the patients who received adjuvant chemotherapy to those treated with 5-fluorouracil-based adjuvant chemotherapy. Patients with metastatic CRC treated with other targeted therapies (i.e. anti-vascular endothelial growth factor or anti-EGFR antibody) were excluded. The patients were followed up until death or December 2012, whichever came first. Informed consent was obtained from all the patients before specimen collection. This study was approved by the respective institutional review boards of the participating institutions. Tumour and paired normal colorectal tissues were reviewed by two pathologists (M.F. and T.H.). The term 'prognostic marker' is used throughout this article according to the REMARK Guidelines (43).

Histopathological evaluation of colorectal serrated lesions

Histological findings for all colorectal serrated lesion specimens were evaluated by a pathologist (M.F.) who was blinded to the clinical and molecular information. Serrated lesions [hyperplastic polyps (HPs) (N = 145), SSA/P (N = 131) and traditional serrated adenoma (TSA) (N = 115)] were classified on the basis of the current World Health Organization (WHO) criteria (44).

RNA extraction and miRNA array analysis

Total RNA was extracted from FFPE tissues using the miRNeasy FFPE Kit (Qiagen, Valencia, CA). The TaqMan® Array Human MicroRNA A + B Cards Set v3.0 (Applied Biosystems, Foster City, CA) was used for simultaneous measurement of the expression of 760 miRNAs on a microfluidic PCR platform. In brief, 1 µg of total RNA was reverse transcribed using the Megaplex Pools Kit (Applied Biosystems), following which miRNAs were amplified and detected by PCR with specific primers and TaqMan probes. PCR was run in the 7900HT Fast Real-Time PCR system (Applied Biosystems), and SDS 2.2.2 software (Applied Biosystems) was used for comparative analysis of the cycle threshold (ΔC_T). U6 snRNA (RNU6B; Applied Biosystems) served as an endogenous control. ΔC_T was calculated by subtracting the C_T values of U6 from the C_T values of the gene of interest. Expression of each miRNA in the tumour samples was calculated using the equation $2^{-\Delta C_T}$, where $\Delta C_T = (C_T\text{miRNA} - C_T\text{U6})$.

Quantitative reverse transcription–PCR of miR-31

MicroRNA-31 (miR-31)-5p expression was analysed using TaqMan microRNA Assays (Applied Biosystems). In brief, 5 ng of total RNA were reverse transcribed using specific stem–loop RT primers, following which they were amplified and detected by quantitative reverse transcription–PCR (qRT–PCR) with specific primers and TaqMan probes. PCR was run in triplicate using the 7500 Fast Real-Time PCR System (Applied Biosystems). SDS v1.4 software (Applied Biosystems) was used for comparative ΔC_T analysis. U6 served as an endogenous control.

DNA extraction, pyrosequencing of KRAS, BRAF *and* PIK3CA *and MSI analysis*

Genomic DNA was extracted from FFPE tissues of colorectal tumours using QIAamp DNA FFPE Tissue Kit (Qiagen). Using extracted genomic DNA, PCR and targeted pyrosequencing were performed for *KRAS* (codons 12 and 13), *BRAF* (*V600E*) and *PIK3CA* (exons 9 and 20) (45). MSI analysis was performed using 10 microsatellite markers, as described previously (46). MSI-high was defined as instability in ≥30% of the markers, and MSI-low/microsatellite stability (MSS) as instability in <30% of the markers (46).

Sodium bisulfite treatment and pyrosequencing to measure MLH1 *promoter methylation*

Bisulfite modification of genomic DNA was performed using a BisulFlash™ DNA Modification Kit (Epigentek, Brooklyn, NY). Bisulfite pyrosequencing for *MLH1* methylation was performed using the PyroMark Kit (Qiagen), as described previously (47).

Colon cancer cell line and miRNA transient transfection

In this study, seven colon cancer cell lines (COLO-320-HSR, DLD-1, HCT-116, HT-29, Lovo, RKO and SW480) were utilized (Supplementary Table 1, available at *Carcinogenesis* Online). Total RNA was extracted from cell pellets using the TRIzol Reagent (Invitrogen by Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Cells were transfected using the Cell Line Nucleofector Kit V (Lonza, Basel, Switzerland) with a Nucleofector I electroporation device (Lonza) for DLD-1, HCT-116 and RKO and Lipofectamine 2000 (Invitrogen by Life Technologies) for COLO-320-HSR, HT-29, Lovo and SW480, according to the manufacturer's instructions. At 72 h after transfection, the cells were harvested for qRT–PCR or western blotting.

Assays for proliferation and invasion

Proliferation of miRNA transfectants was analysed by measuring the uptake of tritiated thymidine in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay; Sigma–Aldrich, St Louis, MO). In brief, transfected cells were seeded into 96-well plates to a density of 5×10^3 cells per well. After incubation for 0, 24, 48, 72 and 96 h, MTT assays were performed using the Cell Counting Kit-8 (Dojindo, Tokyo, Japan) according to the manufacturer's instructions.

Cell invasion was assessed by a Matrigel invasion assay. After incubation for 24 h, 1×10^6 transfected cells suspended in 500 µl of serum-free medium were added to the top of BD BioCoat Matrigel Invasion Chambers (BD Biosciences, Bedford, MA) prehydrated with phosphate-buffered saline, and 750 µl of medium supplemented with 10% fetal bovine serum was added to the lower wells of the plate. After incubation for 24 h, the invading cells were fixed, stained and analysed under a microscope (Olympus, Tokyo, Japan). Cells were counted in five random fields per membrane. In both assays, the experiments with each cell line were performed three times.

Western blot analysis

Protein expression was analysed using a standard immunoblot procedure with anti-KRAS and anti-BRAF. All primary antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-β-actin monoclonal antibody was used as a loading control (Oncogene Research Products, La Jolla, CA). The immunoreactive bands were visualized using enhanced chemiluminescence (Thermo Scientific, Rockford, IL).

Statistical analysis

JMP (version 10) and SAS (version 9) software programs were used for statistical analyses (SAS Institute, Cary, NC). All P values were two sided. Univariate analyses were performed to investigate clinicopathological and molecular characteristics according to the miR-31 expression level; a chi-square test or Fisher's exact test was used for categorical data, whereas analysis of variance was used to compare the mean patient age and tumour size. To account for multiple hypothesis testing in associations between miR-31 expression and other 12 covariates, the P value for significance was adjusted by Bonferroni correction to $P = 0.0042$ (=0.05/12).

In survival analysis, the Kaplan–Meier method and log-rank test were used to assess the survival time distribution. Cox proportional hazards regression models were used to compute mortality hazard ratios (HRs) according to the miR-31 expression status. Stratification by the tumour-node-metastasis disease stage (I, IIA, IIB, IIIA, IIIB, IIIC and IV) was performed using the 'strata' option in the SAS 'proc phreg' command. A multivariate model initially included sex (male versus female), age at diagnosis (continuous), tumour size (continuous), year of diagnosis (continuous), tumour location (proximal colon versus distal colon and rectum), tumour differentiation (well to moderate versus poor), MSI status (MSI-high versus MSS/MSI-low), *MLH1* methylation (present versus absent) and mutations of *BRAF*, *KRAS* and *PIK3CA* (present versus absent). A backward elimination was performed with a threshold of $P = 0.10$, to avoid overfitting. Cases with missing information for any of the categorical covariates [tumour differentiation (1.7%), MSI status (1.9%), *MLH1* methylation (4.9%), mutations of *BRAF* (0.1%), *KRAS* (1.5%) and *PIK3CA* (0.1%)] were included in the majority category of the given covariate to avoid overfitting. We confirmed that excluding cases with missing information in any of the covariates did not substantially alter results (data not shown).

A multivariate logistic regression analysis was employed to examine the associations with miR-31 expression status (as an outcome variable), adjusting for potential confounders. The model initially included a similar set of covariates to the initial Cox model. A backward elimination procedure with a threshold of $P = 0.10$ was used to select variables in the final model. Cases with missing information for a given covariate were included in a majority category in the initial model, and if the covariate remained in the final model, those cases were included using a missing indicator variable in the final model. The P value for significance was adjusted by Bonferroni correction to $P = 0.0042$ (=0.05/12).

Results

Detection of high-level miR-31 expression in BRAF*-mutated CRCs on miRNA array analysis*

To examine the miRNA expression signature in *BRAF*-mutated CRCs, 29 cases of CRCs (Supplementary Table 2, available at *Carcinogenesis* Online) were randomly selected from the CRC specimens for miRNA array analysis. Median levels of expression in the *BRAF* mutation group were compared with those in the *BRAF*

wild-type group (including cases with *KRAS* mutation). miRNA array data revealed differential expression in 33 individual miRNAs ($P < 0.05$ by Mann–Whitney *U*-test) between the two groups (Table I). All 33 miRNAs displayed higher expression levels in the *BRAF* mutation group than in the *BRAF* wild-type group. Of the 760 miRNAs, miR-31-5p was up-regulated the most often (335-fold change, $P = 0.009$).

Distribution of miR-31 expression in CRCs and association of miR-31 with clinicopathological and molecular features

We assayed miR-31 expression in 735 FFPE CRC tissue specimens and successfully obtained 721 (98%) valid results. Fourteen patients were unavailable for miR-31 expression analysis because of the lack of extracted RNA from FFPE CRC tissue specimens. We utilized 721 CRC cases, based on the availability of miR-31-5p expression data. miR-31 expression levels were quantified in CRC specimens and paired normal mucosa specimens. miR-31 expression was calculated using the equation $2^{-\Delta C_T}$, where $\Delta C_T = (C_T \text{ miR-31} - C_T \text{U6})$. To calculate the relative expression of miR-31 in each CRC, $2^{-\Delta C_T}$ of cancer tissue was divided by $2^{-\Delta C_T}$ of paired normal tissue. The distributions of miR-31 expression in the 721 CRC specimens were as follows: mean: 41.9; median: 6.3; SD: 176.2; range: 0.04–2108; interquartile range: 2.0–23.4. Cases with miR-31 expression were then divided into quartiles for further analysis: Q1 (<2.0), Q2 (2.0–6.2), Q3 (6.3–23.3) and Q4 (≥23.4). Table II shows the clinicopathological and molecular features of CRCs according to miR-31 expression level. High miR-31 expression was significantly associated with larger tumour size, proximal location, poor differentiation, advanced disease stage, *BRAF* mutation, *KRAS* mutation and MSI-high status ($P \leq 0.0042$ for all).

High miR-31 expression and patient survival

The influence of high miR-31 expression on clinical outcome was assessed in 721 CRC patients (stages I–IV). During follow-up of the 698 patients eligible for survival analysis, mortality occurred in 149, including 115 deaths confirmed to be attributable to CRCs. The median follow-up time for censored patients was 4.7 years. Kaplan–Meier analysis was performed using categorical variables (Q1, Q2, Q3 or Q4). Significantly higher mortality was observed in patients with high miR-31 expression in terms of cancer-specific survival (log-rank test: $P = 0.0013$) and overall survival (log-rank test: $P = 0.0026$) than in those with low miR-31 expression (Figure 1).

In univariate Cox regression analysis, compared with Q1 cases, significantly higher mortality rates were observed in Q2 cases [HR: 1.96; 95% confidence interval (CI): 1.06–3.77; $P = 0.031$], Q3 cases (HR: 2.16; 95% CI: 1.18–4.13; $P = 0.012$) and Q4 cases (HR: 3.10; 95% CI: 1.76–5.78; $P < 0.0001$) (Table III). Similarly, compared with Q1 cases, an independent association with shorter prognosis was observed in Q4 cases in stage-stratified (HR: 2.49; 95% CI: 1.40–4.67; $P = 0.0016$) and multivariate analyses (HR: 2.91; 95% CI: 1.60–5.57; $P = 0.0004$) for cancer-specific survival (Table III). On the other hand, compared with Q1 cases, slightly but insignificantly higher mortality rates were observed in Q2 and Q3 cases in stage-stratified (Q2: $P = 0.11$, Q3: $P = 0.23$) and multivariate stage-stratified analyses (Q2: $P = 0.14$, Q3: $P = 0.18$) (Table III). Similar results were observed in stage-stratified and multivariate stage-stratified analyses for overall survival (data not shown). Therefore, we made a dichotomous miR-31 expression variable, defining Q4 as the 'high-expression group' and combining Q1, Q2 and Q3 into the 'low-expression group'. In multivariate stage-stratified analysis, compared

Table I. Differentially expressed miRNA in *BRAF*-mutated and *BRAF* wild-type CRCs by miRNA array analysis

No.	Name of miRNA (miR base ID)	Relative miRNA expression (miRNA/U6)			*P*
		BRAF mutation group (median; $N = 7$)	*BRAF* wild-type group (median; $N = 22$)	Fold change (mutation group/wild-type group)	
1	hsa-miR-31-5p	29 925.00	89.30	335.0	0.009
2	hsa-miR-215	7.65	0.10	74.5	0.001
3	hsa-miR-151-3p	1312.00	24.40	53.8	0.003
4	hsa-miR-539-5p	370.00	7.57	48.9	0.021
5	hsa-miR-31-3p	77.30	2.14	36.1	0.002
6	hsa-miR-661	3125.00	91.80	34.1	0.011
7	hsa-miR-197-3p	4.78	0.16	29.2	0.002
8	hsa-miR-483-3p	605.00	21.90	27.6	0.032
9	hsa-miR-185-5p	15.40	0.56	27.3	0.024
10	hsa-miR-223-3p	10.50	0.40	25.9	0.005
11	hsa-miR-451a	23.50	1.00	23.5	0.015
12	hsa-miR-410	19.90	0.85	23.4	0.001
13	hsa-miR-15b-5p	9.01	0.40	22.7	0.004
14	hsa-miR-126-5p	8.45	0.37	22.6	0.013
15	hsa-miR-221-3p	99.90	4.82	20.7	0.048
16	hsa-miR-10b-3p	13.80	0.67	20.6	0.015
17	hsa-miR-29c-3p	19.50	1.02	19.1	0.001
18	hsa-miR-625-5p	185.00	10.30	18.0	0.002
19	hsa-miR-34a-5p	75.70	4.37	17.3	0.032
20	hsa-miR-7-1-3p	10.40	0.60	17.3	0.001
21	hsa-miR-10b-5p	23.10	1.49	15.5	0.008
22	hsa-miR-26b-5p	5.46	0.36	15.2	<0.001
23	hsa-let-7a-5p	5.86	0.42	13.9	0.048
24	hsa-miR-145-3p	2.82	0.20	13.8	0.028
25	hsa-miR-374a-5p	26.30	1.96	13.4	0.001
26	hsa-miR-222-3p	11.80	0.89	13.2	0.011
27	hsa-miR-379-5p	1.41	0.11	13.0	0.024
28	hsa-miR-30c-5p	1.59	0.14	11.8	0.002
29	hsa-miR-100-5p	3.26	0.28	11.6	0.004
30	hsa-miR-625-3p	13.20	1.16	11.4	0.001
31	hsa-miR-142-5p	5.23	0.48	11.0	0.002
32	hsa-miR-99a-5p	2.02	0.19	10.5	0.003
33	hsa-miR-425-5p	10.80	1.07	10.1	0.005

The fold change is expressed as the median of the *BRAF* mutation group divided by that of the wild-type group for each miRNA. *P* values were determined by the Mann–Whitney *U*-test. miRNAs with $P < 0.05$ are listed.

Table II. Clinicopathological and molecular features of 721 CRCs according to quartiles of miR-31 expression

Clinicopathological or molecular feature	Total *N*	miR-31 expression				*P*
		Q1 (<2.0)	Q2 (2.0–6.2)	Q3 (6.3–23.3)	Q4 (≥23.4)	
All cases	721	180	180	181	180	
Gender						
Male	422 (59%)	101 (56%)	107 (59%)	103 (57%)	111 (62%)	0.70
Female	299 (41%)	79 (44%)	73 (41%)	78 (43%)	69 (38%)	
Age (mean ± SD)	66.9 ± 11.4	65.7 ± 11.3	67.3 ± 11.0	67.8 ± 12.0	66.7 ± 11.2	0.34
Tumour size (mm) (mean ± SD)	46.5 ± 23.6	36.6 ± 17.3	44.8 ± 20.9	50.0 ± 23.1	54.4 ± 27.9	<0.0001
Year of diagnosis						
Prior to 2002	334 (46%)	93 (52%)	80 (44%)	76 (42%)	85 (47%)	0.29
2003–2012	387 (54%)	87 (48%)	100 (56%)	105 (58%)	95 (53%)	
Tumour location						
Rectum and distal colon (splenic flexure to sigmoid)	465 (64%)	135 (75%)	125 (69%)	119 (66%)	86 (48%)	<0.0001
Proximal colon (caecum to transverse)	256 (36%)	45 (25%)	55 (31%)	62 (34%)	94 (52%)	
Tumour differentiation						
Well to moderate	655 (92%)	176 (99%)	170 (96%)	162 (91%)	147 (84%)	<0.0001
Poor	54 (8.0%)	2 (1.1%)	7 (4.0%)	16 (9.0%)	29 (16%)	
Disease stage						
I	138 (19%)	59 (32%)	31 (17%)	23 (13%)	25 (14%)	<0.0001
IIA	157 (22%)	46 (26%)	42 (23%)	37 (20%)	32 (18%)	
IIB	57 (7.9%)	10 (5.6%)	10 (5.6%)	20 (11%)	17 (9.4%)	
IIIA	41 (5.7%)	14 (7.8%)	11 (6.1%)	12 (6.6%)	4 (2.2%)	
IIIB	163 (23%)	23 (13%)	45 (25%)	46 (25%)	49 (27%)	
IIIC	82 (11%)	14 (7.8%)	20 (11%)	21 (12%)	27 (15%)	
IV	83 (12%)	14 (7.8%)	21 (12%)	22 (12%)	26 (14%)	
BRAF mutation						
Wild-type	685 (95%)	176 (98%)	179 (99%)	173 (96%)	157 (87%)	<0.0001
Mutant	35 (4.9%)	3 (1.7%)	1 (0.6%)	8 (4.4%)	23 (13%)	
KRAS mutation						
Wild-type	479 (67%)	130 (74%)	123 (69%)	126 (70%)	100 (57%)	0.0042
Mutant	231 (33%)	45 (26%)	56 (31%)	54 (30%)	76 (43%)	
PIK3CA mutation						
Wild-type	642 (89%)	168 (94%)	160 (89%)	163 (90%)	151 (84%)	0.023
Mutant	78 (11%)	11 (6.2%)	20 (11%)	18 (9.9%)	29 (16%)	
MSI status						
MSS/MSI-low	658 (93%)	172 (99%)	171 (97%)	164 (92%)	151 (85%)	<0.0001
MSI-high	49 (6.9%)	2 (1.2%)	6 (3.4%)	14 (7.9%)	27 (15%)	
MLH1 methylation						
Unmethylated	387 (56%)	106 (62%)	102 (59%)	94 (54%)	85 (51%)	0.15
Methylated	299 (44%)	65 (38%)	71 (41%)	80 (46%)	83 (49%)	

Percentage (%) indicates the proportion of cases with a specific clinicopathological or molecular feature within a given quartile category (Q1, Q2, Q3 or Q4) of miR-31 expression by qRT–PCR. *P* values were calculated by analysis of variance for age and tumour size and by a chi-square test or Fisher's exact test for all other variables. To account for multiple hypothesis testing in associations between miR-31 expression and other 12 covariates, the *P* value for significance was adjusted by Bonferroni correction to $P = 0.0042$ (=0.05/12).

with the 'low-expression group', a significantly higher mortality rate was observed in the 'high-expression group' (HR: 2.06; 95% CI: 1.36–3.09; $P = 0.0008$) in cancer-specific analysis (Table III).

In stage-stratified (stages I–IV) analysis, the mortality rate in terms of cancer-specific survival was significantly higher in CRC groups (stages II–IV) with high miR-31 expression levels (log-rank test: $P = 0.035$, $P = 0.020$ and $P = 0.024$, respectively) than in those with low miR-31 expression levels (Supplementary Figure 1, available at *Carcinogenesis* Online). Our data also showed that high miR-31 expression was related to cancer-specific mortality, regardless of *BRAF* status (Supplementary Figure 2, available at *Carcinogenesis* Online).

Multivariate logistic regression analysis in cases of high miR-31 expression

Considering potential confounding and potential cause-effect sequence, we performed a multivariate logistic regression analysis to assess the relationships with miR-31 expression. The results showed that high miR-31 (Q4) expression was significantly associated with *BRAF* [odds ratio (OR): 7.05; 95% CI: 3.08–16.8; $P < 0.0001$], *KRAS* mutation (OR: 2.61; 95% CI: 1.75–3.90; $P < 0.0001$) and tumour location in the proximal colon (OR: 2.21; 95% CI: 1.49–3.28; $P < 0.0001$) (Table IV).

Association of miR-31 expression and clinicopathological and molecular features in serrated lesions

We assessed 650 FFPE tissue specimens of serrated lesions and non-serrated adenomas in the miR-31 expression assay and successfully obtained 632 (97%) valid results. Then miR-31 expression levels were also quantified in 381 colorectal serrated lesions and 251 non-serrated adenomas. It is very difficult to obtain miRNA specimens of paired normal tissue for comparison with colorectal serrated lesions and non-serrated adenomas by endoscopic resection

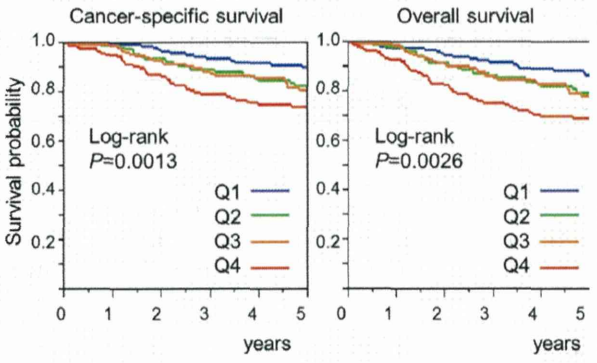

Fig. 1. Kaplan–Meier survival curves for CRCs (stages I–IV; $N = 698$) according to the miR-31 expression level. Cancer-specific survival: left panel and overall survival: right panel. Significantly higher mortality rates were observed in patients with high miR-31 expression than in those with low expression for both cancer-specific (log-rank test: $P = 0.0013$) and overall survival (log-rank test: $P = 0.0026$).

because of the small size of the resected sample. Therefore, we incorporated pooled normal mucosa specimens in each plate to standardize all assay runs. Distributions of miR-31 expression were as follows (mean ± SD; median): HP (10.3 ± 19.3; 2.6), SSA/P (20.5 ± 25.1; 12.9), TSA (24.8 ± 28.0; 14.7) and non-serrated adenoma (14.1 ± 24.9; 3.4).

Supplementary Table 3, available at *Carcinogenesis* Online, shows the clinicopathological and molecular features, including miR-31 expression, in serrated lesions and non-serrated adenomas. High miR-31 expression (Q4 in CRCs; expression level ≥23.4) was frequently detected in cases of SSA/P [32% (41/128)] and TSA [37% (42/113)] compared with those of HP [13% (18/140)] and non-serrated adenoma [19% (45/251)] ($P < 0.0001$). Multivariate regression analysis was adjusted for potential confounders including *BRAF* and *KRAS* mutations, tumour location and tumour size. The results showed a persistent significant association between high miR-31 expression and histological type [SSA/P: $P = 0.0092$, TSA: $P < 0.0001$ (HP as a referent)].

Functional analysis of miR-31 expression in colon cancer cell lines

miR-31 mimics and the inhibitor were transfected into colon cancer cell lines. The results confirmed the up-regulation or down-regulation of miR-31 expression (Supplementary Figure 3, available at *Carcinogenesis* Online). The Matrigel invasion assay revealed enhanced invasive potential of the miR-31 mimic (Figure 2A) after transfection (72 h later) into cancer cell lines. Similar results were observed in the proliferation assay (data not shown). In the proliferation assay, significantly decreased cell proliferation was also observed as a result of transfection (96 h later) of the miR-31 inhibitor (Figure 2B). Similar results were observed in the invasion assay (data not shown).

To determine the effect of miR-31 on *BRAF* and *KRAS* target proteins, expression of those proteins was compared before and after transfection (72 h later) of the miR-31 inhibitor into the cell lines. The results of western blot analysis demonstrated that after transfection, *BRAF* target proteins decreased in colon cancer cell lines, regardless of the mutational status (Figure 2C). In contrast, none of the colon cancer cell lines showed a decrease in *KRAS* target proteins.

Discussion

In this study, specific miRNA expression associated with *BRAF* (*V600E*) mutation was identified. The results of miRNA array analysis revealed that miR-31 was the most up-regulated gene in *BRAF*-mutated CRCs compared with *BRAF* wild-type CRCs. In a database of 721 patients with CRC, high miR-31 expression was associated with *BRAF* and *KRAS* mutations and proximal location in multivariate logistic regression analysis. After the transfection of the miR-31 inhibitor, western blot analysis revealed a decrease in BRAF target protein in colon cancer cell line. Thus, our data support the hypothesis

Table III. Association of miR-31 expression with patient mortality in CRCs

miR-31 expression (quartile)	Total *N*	Cancer-specific survival		
		Univariate	Stage-stratified	Multivariate stage-stratified
		HR (95% CI)	HR (95% CI)	HR (95% CI)
Q1 (<2.0)	171	1 (referent)	1 (referent)	1 (referent)
Q2 (2.0–6.2)	174	1.96 (1.06–3.77)	1.65 (0.89–3.18)	1.59 (0.86–3.07)
Q3 (6.3–23.9)	177	2.16 (1.18–4.13)	1.46 (0.79–2.81)	1.53 (0.83–2.96)
Q4 (≥24.0)	176	3.10 (1.76–5.78)	2.49 (1.40–4.67)	2.91 (1.60–5.57)
P for trend		0.0009	0.013	0.0032
Low-expression group (Q1–3)	522	1 (referent)	1 (referent)	1 (referent)
High-expression group (Q4)	176	1.84 (1.25–2.67)	1.78 (1.20–2.60)	2.06 (1.36–3.09)
P		0.0024	0.0043	0.0008

The multivariate, stage-stratified Cox model included the miR-31 expression variable stratified by sex, age at diagnosis, tumour size, year of diagnosis, tumour location, tumour differentiation, MSI status, *MLH1* methylation and mutations of *BRAF*, *KRAS* and *PIK3CA*.

Table IV. Multivariate logistic regression analysis of miR-31 expression in CRCs

Variables in the final model for miR-31 expression (as an outcome variable) [high-expression group (Q4) versus low-expression group (Q1–3)]	Adjusted OR (95% CI)	*P*
BRAF mutant (versus wild-type)	7.05 (3.08–16.8)	<0.0001
KRAS mutant (versus wild-type)	2.61 (1.75–3.90)	<0.0001
Proximal colon (versus distal colon and rectum)	2.21 (1.49–3.28)	<0.0001
Poor differentiation (versus well to moderate)	2.75 (1.38–5.44)	0.0044
Tumour size (for 30 mm increase as a unit)	1.46 (1.11–1.92)	0.0062

A multivariate logistic regression analysis assessing the relationships with miR-31 expression status initially included sex, age, tumour size, year of diagnosis, tumour location, tumour differentiation, disease stage, MSI, *MLH1* methylation, and mutations of *BRAF*, *KRAS* and *PIK3CA*, considering potential confounding and causal relationships. For multiple hypothesis testing, the *P* value for significance was adjusted by Bonferroni correction to 0.0042 (=0.05/12).

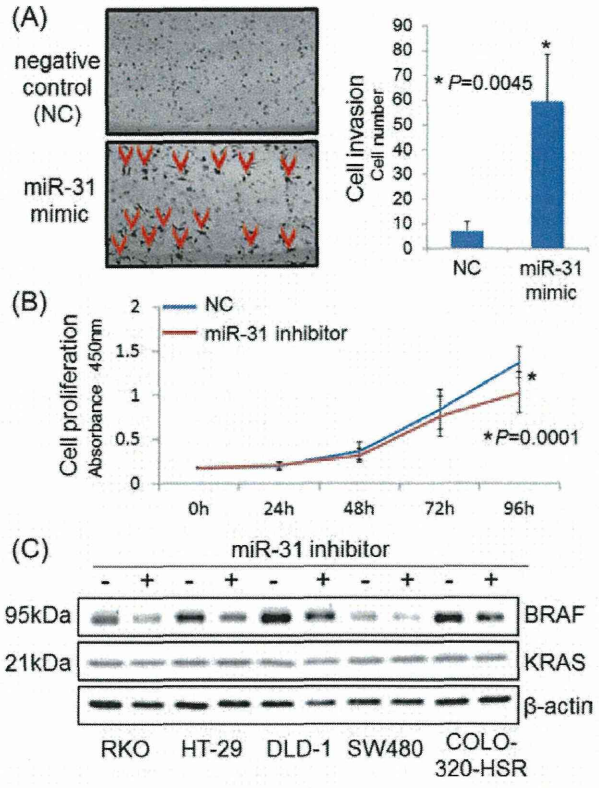

Fig. 2. Functional analysis of miR-31. (**A**) Results of the Matrigel invasion assay. Invading cells are indicated by arrow heads in the left panel. The right panel represents the means of five random microscopic fields per membrane; error bars represent the standard deviations. This assay revealed that the miR-31 mimic enhanced invasion by 8.5-fold ($P = 0.0045$) in SW480 cells (*KRAS* mutated) after transfection (72h later). The P value was analysed using a paired T-test. (**B**) In the proliferation assay, the miR-31 inhibitor significantly decreased cell proliferation in HT-29 cells (*BRAF* mutated) ($P = 0.0001$). The graph depicts the means of 16 replications; error bars represent standard deviations. (**C**) In western blot analysis, after transfection (72h later) of the miR-31 inhibitor, *BRAF* target proteins were decreased in RKO cells (*BRAF* mutated), HT-29 cells (*BRAF* mutated), DLD-1 cells (*KRAS* mutated), SW480 cells (*KRAS* mutated) and COLO-320-HSR (wild-type), respectively.

that miR-31 may regulate *BRAF* activation in CRCs. We also identified that high miR-31 expression was an unfavourable prognostic factor in patients with CRC, independent of clinicopathological and molecular features. In contrast, high miR-31 expression was frequently detected in cases with SSA/P and TSA compared with those with HP, suggesting an oncogenic role of this miRNA in the serrated pathway. The transfection of the miR-31 inhibitor exhibited an antitumour effect in functional analysis. Therefore, miR-31 may be a promising diagnostic biomarker and the therapeutic target in patients with CRC.

miR-31 is located at 9p21.3 and is reportedly deregulated in various human cancers (8,9,11,12,15). Previous studies have shown that miR-31 has oncogenic potential in oesophageal squamous cell carcinoma (8) and acts as a tumour suppressor in oesophageal adenocarcinoma (15), gastric cancer (12), ovarian cancer (11) and breast cancer (9). With regard to CRC, an association has been reported between miR-31, oncogenic potential (21–24,26,48), deeper invasion (24,48) and advanced disease stage (21,24,48); however, none of these studies have examined the association between miR-31 expression and mortality in CRC patients. In the present study, a large database was utilized. High miR-31 expression was independently associated with shorter prognosis in the multivariate stage-stratified Cox model. The importance of large-scale studies cannot be emphasized enough. Small studies with null results are much less likely to remain unpublished compared with small studies with significant results; this leads to publication bias. In contrast to previous studies (21–24,26,48), the present study examined the miR-31 expression status in a much larger sample of CRCs. Therefore, our data support the hypothesis that high miR-31 expression may be a prognostic biomarker of CRC.w Nevertheless, the data on CRCs presented here have some limitations, including the cross-sectional, observational nature of the study. Future independent studies should confirm the correlation between miR-31 and unfavourable prognosis in patients with CRC.

The tumour molecular characterization for personalized medicine is becoming important in CRCs (1–5,16,17,45). Accumulating evidence suggests that similar to *PIK3CA* and *PTEN* mutations, *BRAF* mutations confer therapeutic resistance to cetuximab and panitumumab (2,3) in patients with CRCs because these genes are located downstream of EGFR. In addition, a relationship between *BRAF* mutation and unfavourable survival has been previously reported in patients with CRCs (2,5). These results suggest that *BRAF* mutation can be a new biomarker for molecular diagnosis and identification of prognostic factors; however, no previous study has identified specific miRNAs associated with *BRAF* mutation in a large sample of colorectal tumours. In the current study, associations were identified between miR-31 expression and *BRAF* mutation and CRC prognosis. Previous studies have detected high miR-31 expression in CRC patients with MSI-high status (20,28,34) or poor differentiation (21,23). In the present study, high miR-31 expression was significantly associated with MSI-high status in univariate analysis; however, no significant association was observed in multivariate analysis. Furthermore, in serrated lesions, despite the fact that MSI-high was quite low [1.5% (6/381)], high miR-31 expression was frequently detected in cases with SSA/P and TSA. Thus, high miR-31 expression in CRC patients with MSI-high in previous studies (20,28,34) may have been due to *BRAF* mutation, which has been strongly associated with MSI-high status (1,5).

Recent studies have reported that several miRNAs target the genes in the downstream effectors of EGFR, such as miR-143 and miR-145 (25,29) for *KRAS*, miR-520a and miR-525 (30) for *PIK3CA* and miR-21 (31,32) and miR-155 (32) for *PTEN*. Moreover, *BRAF* is thought to be targeted by miR-143 and miR-145, which play a role as tumour suppressors (29). In the present study, high miR-31 expression was strongly associated with *BRAF* mutation in a CRC large sample. In addition, after transfection of the miR-31 inhibitor, western blot analysis revealed a decrease in *BRAF* target protein. These results support the hypothesis that miR-31 may regulate the activation of *BRAF* gene in CRC. The exact mechanism of this regulation by miR-31 remains unknown; however, a recent study has reported that miR-31 may target a RAS p21 GTPase-activating protein 1 (RASA1), which is a negative regulator of the RAS–RAF–MEK–ERK signalling pathway (35). Therefore, miR-31 may regulate *BRAF* activity via suppression of RASA1 in CRC, resulting in up-regulation of the signalling pathway. These findings also imply that miR-31 may serve as a molecular target of the RAF or MEK inhibitor.

Our data also showed a decrease in *BRAF* target proteins regardless of the mutational status after transfection of the miR-31 inhibitor. This decrease in *BRAF* target proteins was observed in all cell lines; however, none of the colon cancer cell lines exhibited a decrease in *KRAS* target protein. One possible explanation for these phenomena is that miR-31 may target the negative regulator, which plays a role in the pathways downstream of RAS. Further functional analysis is required to clarify the regulatory role of miR-31 in the RAS–RAF–MEK–ERK signalling pathway and its potential as a molecular target of those inhibitors.

Previously, SSA/P was often classified as HP, which was considered to have no malignant potential. However, recent studies have shown that SSA/P is mainly observed in the proximal colon (49) and is associated with frequent *BRAF* mutation and *MLH1* methylation (40–42). These results suggest that SSA/P possesses malignant potential and might be a precursor lesion of MSI-high CRC with *BRAF* mutation in the proximal colon. With regard to miRNA expression in serrated lesions, a previous study involving serrated lesions ($N = 37$) reported that SSA/P was characterized by high levels of miR-181b

and miR-21 expression compared with HP (50). However, the authors concluded that discrimination between the two lesions was impossible on the basis of miR-181b and miR-21 expression. Thus, the effects of miRNA expression in serrated lesions remain largely unknown. In the present study, high miR-31 expression was frequently detected in cases with SSA/P and TSA compared with those with HP in large samples of serrated lesions ($N = 381$). After adjusting for *BRAF* and *KRAS* mutation status, tumour location and tumour size, a persistently significant association between high miR-31 expression and the pathological features of SSA/P and TSA was observed. Thus, our data suggest that high miR-31 expression may occur in the early stage of colorectal tumorigenesis and play an oncogenic role in serrated lesions. Moreover, our findings challenge the common conception of discrete molecular features of SSA/Ps versus HPs. They may, therefore, have a substantial impact on clinical and translational research.

In conclusion, in this study, high miR-31 expression was associated with *BRAF* mutation involving a CRC large sample. This result may indicate that miR-31 is one of the important miRNAs in CRC with *BRAF* mutation. In addition, high miR-31 expression was associated with patient mortality. Finally, an antitumour effect was observed as a result of transfection of the miR-31 inhibitor. Thus, miR-31 may be a promising diagnostic biomarker and therapeutic target in patients with CRC. Moreover, our data suggest that miR-31 may play an important role in the progression of serrated lesions.

Supplementary material

Supplementary Tables 1–3 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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Hepatic biliary epithelial cells acquire epithelial integrity but lose plasticity to differentiate into hepatocytes *in vitro* during development

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Summary

In developing organs, epithelial tissue structures are mostly developed by the perinatal period. However, it is unknown whether epithelial cells are already functionally mature and whether they are fixed in their lineage. Here we show that epithelial cells alter their plasticity during postnatal development by examining the differentiation potential of epithelial cell adhesion molecule $(EpCAM)^+$ cholangiocytes (biliary epithelial cells) isolated from neonatal and adult mouse livers. We found that neonatal cholangiocytes isolated from 1-week-old liver converted into functional hepatocytes in the presence of oncostatin M and Matrigel®. In contrast, neither morphological changes nor expression of hepatocyte markers were induced in adult cholangiocytes. The transcription factors hepatocyte nuclear factor 4α and CCAAT/enhancer binding protein α (C/EBPα), which are necessary for hepatocytic differentiation, were induced in neonatal cholangiocytes but not in adult cells, whereas grainyhead-like 2 (Grhl2) and hairy-enhance of slit 1 (Hes1), which are implicated in cholangiocyte differentiation, were continuously expressed in adult cells. Overexpression of C/EBPα and Grhl2 promoted and inhibited hepatocytic differentiation, respectively. Furthermore, adult cholangiocytes formed a monolayer with higher barrier function than neonatal ones did, suggesting that cholangiocytes are still in the process of epithelial maturation even after forming tubular structures during the neonatal period. Taken together, these results suggest that cholangiocytes lose plasticity to convert into hepatocytes during epithelial maturation. They lose competency to upregulate hepatocytic transcription factors and downregulate cholagiocytic ones under conditions inducing hepatocytic differentiation. Our results suggest that a molecular machinery augmenting epithelial integrity limits lineage plasticity of epithelial cells.

Key words: Epithelial progenitors, Plasticity, Cholangiocytes, Bile duct, Mature hepatocytes

Introduction

During development, tissue stem/progenitor cells differentiate to multiple types of epithelial cells, which establish various tissue structures, including alveoli in the lung, renal tubules in the kidney, and hepatic cords and bile ducts in the liver. Given that organs need to perform their physiological functions after the birth, epithelial tissue structures may be mostly developed at the birth or soon after. However, it is unknown whether epithelial cells are fixed in their lineage and fully functional in neonatal organs.

The liver contains two types of epithelial cells, named hepatocytes and cholangiocytes, which originate from hepatoblasts (fetal liver stem/progenitor cells) during development (Oertel et al., 2003; Tanimizu et al., 2003). Cholangiocytes are biliary epithelial cells forming bile duct tubules. Bile ducts connect the liver to the intestine to drain the bile secreted by hepatocytes. It can be assumed that cholangiocytes acquire epithelial characteristics including secretory and barrier functions when they establish the tubular structure, since it is physiologically important to modulate the composition of bile and avoid any leakage of bile during drainage. However, it is unknown whether cholangiocytes in the neonatal liver have similar epithelial characteristics as those in the adult liver.

In the adult liver, there are at least three possible sources of hepatocytes and cholangiocytes: self-duplication of mature cells, the stem cell system, and lineage conversion. The self-duplication of hepatocytes and cholangiocytes to replace aged or damaged cells is the simplest way, which may be the case in normal and in acutely injured livers (Michalopoulos, 2007; Malato et al., 2011). In contrast, after severe chronic liver injury, the duplication ability of the epithelial cells may be exhausted and stem or progenitor cells may be activated to supply hepatocytes and cholangiocytes (Espanol-Suner et al., 2012). In addition to the self-duplication and stem/progenitor cell systems, lineage conversion should be taken into consideration (Michalopoulos, 2011). It has been shown that mature hepatocytes (MHs) have the potential to transdifferentiate into cholangiocyte-like cells (Nishikawa et al., 2005; Zong et al., 2009). In contrast to hepatocytes, it remains unclear whether cholangiocytes have the ability to convert into hepatocytes.

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In this work we examined the differentiation potential of cholangiocytes in neonatal and adult mouse liver. We found that neonatal, but not adult, epithelial cell adhesion molecule $(EpCAM)^+$ cholangiocytes expressed hepatocytic transcription factors and converted into hepatocytes *in vitro* that were structurally and functionally similar to MHs. Interestingly, neonatal cholangiocytes are still immature compared with adult ones even though they have already established tubular structures *in vivo*. Our results indicate that neonatal cholangiocytes possess plasticity to convert into hepatocytes but lose this ability during maturation of bile ducts. We further demonstrated that a transcription factor implicated in epithelial maturation limited lineage plasticity of cholangiocytes.

Results

Cholangiocytes proliferate and retain the cholangiocytic phenotype on type I collagen gel

Because the number of cholangiocytes isolated from the liver is limited and not enough to examine their differentiation potential, we first established a primary culture in which cholangiocytes keep the original characteristics and efficiently proliferate. To isolate mature cholangiocytes from 6-week-old (6W) mouse liver, two-step collagenase perfusion was performed and the remaining tissue containing Glisson's capsules was further digested. $EpCAM^+$ cholangiocytes were enriched by magnetic-activated cell sorting (MACS; supplementary material Fig. S1). They were plated on culture wells coated with type I collagen (Col-I) or a thin layer of Matrigel (MG), or covered with Col-I gel or MG gel (Fig. 1A). On wells coated with Col-I, only a very small number of cells survived and proliferated. On MG-coated or MG gel wells, 2 or 3 days after plating, cells began to proliferate slowly. On Col-I gel, cells proliferated very efficiently. In all four conditions, cells survived and proliferated after replating at day 7 of primary culture. Importantly, on Col-I gel, as well as MG gel, expression of EpCAM was retained on cholangiocytes but disappeared when grown in wells coated with Col-I (Fig. 1B; supplementary material Fig. S2). During the culture on Col-I gel, cholangiocytes maintained expression of cholangiocyte markers [osteopontin (OPN), SRY-related HMG box transcription factor 9 (Sox9) and hepatocyte nuclear factor (Hnf)1β], but did not express the hepatocyte marker, Hnf4α (Fig. 1C), and kept epithelial characteristics, such as the ability to form cystic structures in 3D culture; about 1% of cells formed cysts during 10 days in culture ever after the fourth passage (Fig. 1D; supplementary material Fig. S2). We further confirmed that, like mouse cholangiocytes, human $EpCAM^+$ cholangiocytes proliferated and retained the expression of EpCAM on Col-I gel (supplementary material Fig. S3). In the following experiments, we examined differentiation potential of cholangiocytes after expansion in Col-I gel culture.

Hepatocytic differentiation potential of adult cholangiocytes

To examine the hepatocytic differentiation potential of cholangiocytes, $EpCAM^+$ cells derived from 6–8W mouse livers were cultured on Col-I gel for 5 days and then replated onto dishes coated with gelatin. To induce hepatocytic differentiation, oncostatin M (OSM) was added to the culture medium after the cells reached confluency. On day 9 in culture,

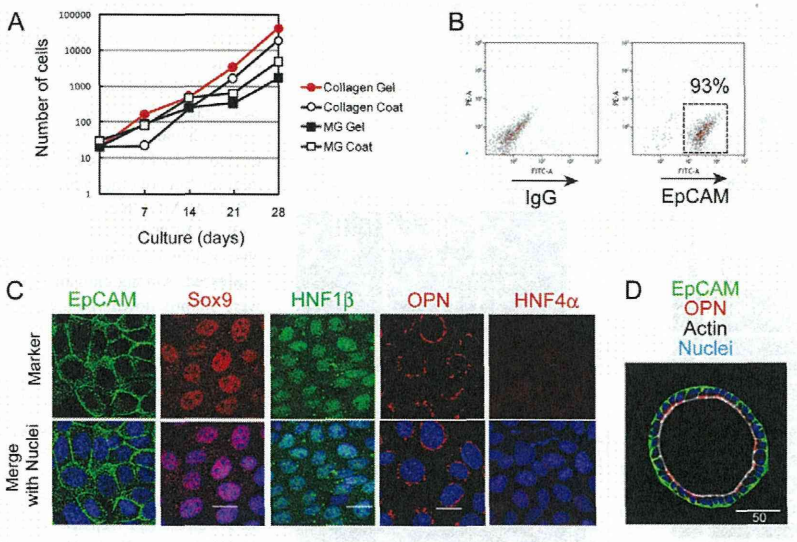

Fig. 1. *In vitro* expansion of $EpCAM^+$ cholangiocytes on Col-I gel. (A) Proliferation of $EpCAM^+$ cholangiocytes on Col-I and Matrigel®. Cholangiocytes were cultured on Col-I-coated or MG-coated wells or on Col-I gel or MG. Every 7 days, they were replated onto dishes coated with the same extracellular matrix as the primary culture. During primary culture, cholangiocytes proliferated on Col-I gel, MG gel and MG-coated dishes, though they proliferated most efficiently on Col-I gel. Beyond secondary culture, cholangiocytes proliferated in all conditions. (B) Adult cholangiocytes retained the expression of EpCAM on Col-I gel. EpCAM expression was examined by fluorescence-activated cell sorting (FACS). More than 90% of cells retained EpCAM expression on Col-I gel. (C) Adult cholangiocytes retained the expression of marker genes on Col-I gel. Cultured cholangiocytes expressed the cholangiocyte markers EpCAM, Sox9, HNF1β, and OPN. $EpCAM^+$ cells isolated from 6W mouse liver were cultured on Col-I gel for 7 days, fixed in 4% PFA, and incubated with anti-Sox9, anti-HNF1β and anti-OPN antibodies. Nuclei were counterstained with Hoechst 33258. (D) Adult cholangiocytes form cysts with the central lumen in three-dimensional culture. At day 7, cultured cholangiocytes were dissociated from Col-I gel, replated on a layer of MG, and then overlaid with 5% MG. Cysts were stained with anti-EpCAM (green), anti-OPN (red), and phalloidin (white). Nuclei were counterstained with Hoechst33258 (blue).

cells were overlaid with 5% MG (Fig. 2A). Dense cytoplasm and clear cell–cell contacts were observed after sequential treatment with OSM and MG (Fig. 2B). However, as shown in Fig. 2B, the cells barely expressed hepatocyte markers including albumin, carbamoylphosphate synthetase I (CPSI), phosphoenolpyruvate carboxykinase (PEPCK), and tryptophan 2,3-dioxygenase (Tdo2). Thus, hepatocytic characteristics could not be induced in adult cholangiocytes.

Hepatocytic differentiation potential of neonatal cholangiocytes

To investigate whether cholangiocytes have the potential to differentiate into hepatocytes during the early stage of bile duct formation, we applied the same culture conditions to neonatal cholangiocytes isolated from 1W liver. Similar to adult cholangiocytes, neonatal cholangiocytes continued to express cholangiocyte markers during culture on Col-I gel (supplementary material Fig. S4). As shown in Fig. 2A, neonatal cells cultured on gelatin proliferated and formed a monolayer in which the cells were in close contact with each other. After addition of OSM to the medium on day 5, the cells altered their morphology, developing round nuclei and dense cytoplasm. When the cells were overlaid with MG, cytoplasmic granularity increased. Furthermore, bile canaliculus (BC)-like structures were observed between the cells. During the sequential treatment of OSM and MG, there was increased expression of the genes for albumin, metabolic enzymes including glucose 6-phosphatase (G6Pase), PEPCK, tyrosine aminotransferase (TAT), Tdo2, CPSI and cytochrome P450 proteins (Cyps) (Fig. 2B). We also examined expression of cholangiocyte markers including cytokeratin (CK) 7, CK19 and EpCAM, and found that CK7 and EpCAM were downregulated during hepatocytic differentiation (Fig. 2B and supplementary material Fig. S5). Immunocytochemical analysis showed that albumin and CPSI proteins, which were not expressed in neonatal cholangiocytes at the beginning of the culture period, were expressed in the cytoplasm after inducing hepatocytic differentiation (Fig. 2C3; Figs 4, 7, 8), whereas both proteins were not induced in adult cholangiocytes (Fig. 2C1; Figs 2, 5, 6). However, EpCAM was not downregulated in adult cholangiocytes but was in neonatal ones during culture (Fig. 2C9–12). To examine whether cells treated with MG acquired differentiated functions, ammonium chloride was added to the culture medium. The concentration of ammonium ions in the medium gradually decreased with the time in the wells of cultured neonatal cholangiocytes but not in those of adult cells

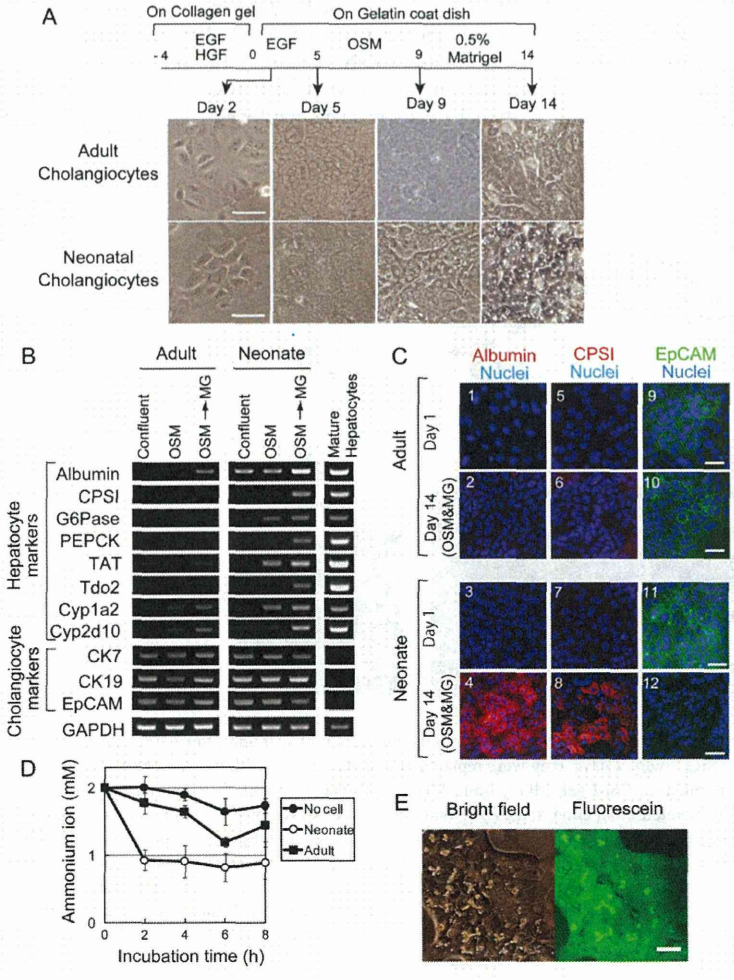

Fig. 2. Neonatal, but not adult, cholangiocytes differentiate to functional hepatocytes. (A) Morphological changes of adult and neonatal cholangiocytes during culture. Adult cholangiocytes show dense cytoplasm at day 5 in culture. Cell–cell contacts were clearly visible after overlaying with MG. Neonatal cholangiocytes had round nuclei and dense cytoplasm in the presence of OSM. Cell–cell contacts were more evident after overlaying with MG. After expansion on Col-I gel, adult and neonatal cholangiocytes were used to induce hepatocytic characteristics by sequentially treating them with OSM and MG. Scale bars: 50 μm. (B) Neonatal cholangiocytes were induced to express hepatocyte markers. Hepatocyte marker expression was examined by PCR. Adult cholangiocytes weakly expressed albumin but not other hepatocyte markers even in the presence of OSM and MG. In contrast, hepatocyte markers such as CPSI, G6Pase, PEPCK, TAT and Tdo2 were induced in neonatal cholangiocytes during culture. Cyp1a2 and Cyp2d10 were also expressed. Among cholangiocyte markers, CK7 and EpCAM were slightly downregulated in the presence of OSM and MG. Experiments were repeated three times, independently, and the representative data are shown. (C) Expression of hepatocyte markers at the protein level. At 1 day after plating onto gelatin-coated dishes, neonatal cholangiocytes did not express albumin and CPSI. After inducing hepatocytic differentiation, albumin (red) was expressed in many cells. Some cells expressed CPSI (red). In contrast, both proteins were not expressed in adult cholangiocytes before and after treatment of OSM and MG. Scale bars: 50 μm. (D) Hepatocytes derived from neonatal cholangiocytes eliminated ammonium ions from the medium. Ammonium chloride (2 mM) was added to neonatal cholangiocytes treated with MG. Ammonium ions in the medium were eliminated by hepatocytes derived from neonatal cholangiocytes. Average values at each time point are shown (±s.d.). (E) Hepatocytes derived from neonatal EpCAM^{+} cells formed BC-like structures. After incubation in the presence of MG, cells were further treated with 100 μM taurocholate, and FDA was then added. Hepatocytes derived from neonatal cholangiocytes metabolized FDA and fluorescein was secreted into BC-like structures. Scale bar: 50 μm.