CR

Table 1. Patient characteristics.

	Group α2a	Group α2b	P value
Number	61	88	
% of retreatment cases	41.0%	25.0%	NS
Gender (male/female)	30/31	42/46	NS
Age (years)	58.3±8.7	57.6±10.5	NS
Body mass index (kg/m^2)	22.4±2.1	23.5±2.5	NS
HCV RNA (log IU/mL)	6.1±0.5	6.2±0.6	NS
AST (IU/L)	51.0±26.5	55.5±40.3	NS
ALT (IU/L)	64.8±40.7	69.8±66.8	NS
GGT (IU/L)	54.4±64.2	52.2±54.8	NS
Hemoglobin (g/dL)	14.0±1.4	13.8±1.4	NS
White blood cell (/μL)	5.008±1.320	5.002±1.374	NS
Platelet (×10^4/μL)	16.1±4.7	19.1±9.0	NS

AST – aspartate aminotransferase; ALT – alanine aminotransferase; GGT – γ-glutamyl transpeptidase; NS – not significant.

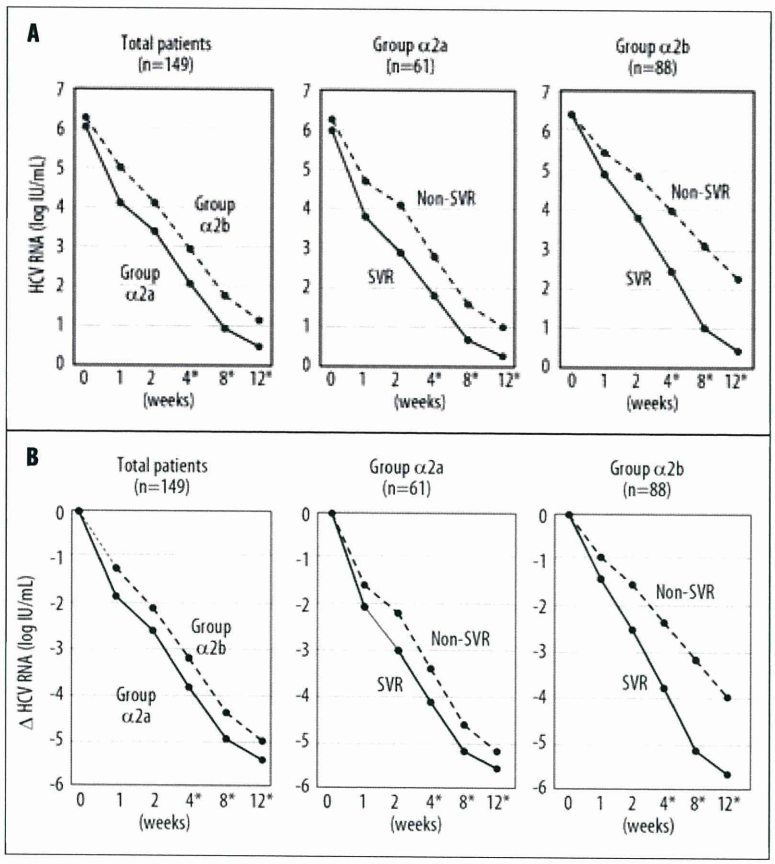

Figure 2. (**A**) Serum HCV RNA values in patients with chronic hepatitis C during peg-IFN plus ribavirin combination therapy. The levels were compared between group α2a and group α2b (left panel), between SVR and non-SVR patients in group α2a (center panel), and between SVR and non-SVR patients in group α2b (right panel). * $p<0.01$, ** $p<0.05$. (**B**) Levels of viral decline (HCV RNA) from baseline in patients with chronic hepatitis C during peg-IFN plus ribavirin combination therapy. Levels were compared between group α2a and group α2b (left panel), between SVR and non-SVR patients in group α2a (center panel), and between SVR and non-SVR patients in group α2b (right panel). * $p<0.01$, ** $p<0.05$.

vs. 6.2±0.6 log IU/mL, respectively). SVR rate was lower in group α2a (54.1%) than in group α2b (61.4%), although the difference was not statistically significant (data not shown).

HCV RNA concentrations declined earlier in group α2a, and group α2a showed significantly lower concentrations than group α2b at weeks 4, 8, and 12 after starting treatment (Figure 2A). In both groups, HCV RNA levels were significantly lower at weeks 1, 2, 4, 8, and 12 in SVR patients compared with non-SVR patients (Figure 2A). The level of viral decline to baseline levels (net viral decline) was significantly greater at weeks 4, 8, and 12 in group α2a than in group α2b (Figure 2B). The level of viral decline in SVR patients was significantly greater than that in non-SVR patients at

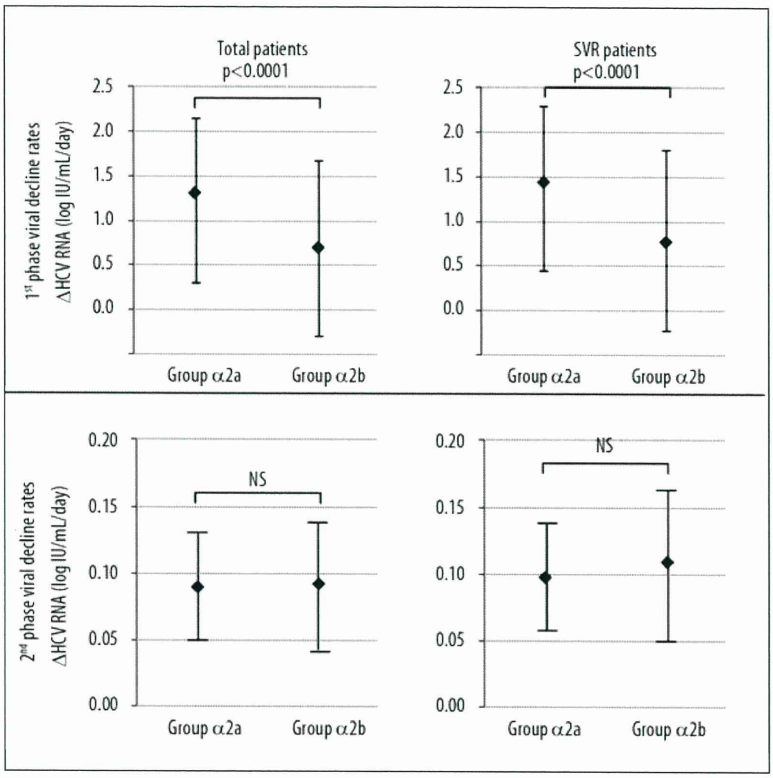

Figure 3. Comparison of first- and second-phase decline rates between group α2a and group α2b in total patients (left panels) and in SVR patients (right panels). Diamonds with lines indicate means ± standard deviation.

week 2 in group α2a, whereas this was evident at all assessment time-points in group α2b (Figures 2B).

In both the total population and SVR patients, first-phase viral decline rates were significantly higher in group α2a compared with group α2b (1.31±0.84 *vs.* 0.70±0.97 log IU/mL/day in total population, p<0.0001; 1.45±0.85 *vs.* 0.78±1.0 log IU/mL/day in SVR patients, p<0.0001) (Figure 3, upper panels). On the other hand, second-phase viral decline rates were similar in the 2 groups (Figure 3, lower panels).

Discussion

As shown in Table 1, no significant differences were found in sex, age, viral load, body weight, platelet counts, or biochemical analysis that would influence the response to antiviral treatments. Retreatment patients were included in this study; their percentage was higher in group α2a than in group α2b (41.0% *vs.* 25.0%), but the difference was not significant. Of note, previous treatments in all retreatment patients were unmodified IFN monotherapy, which is generally ineffective for patients with genotype 1 HCV (SVR rate <5%). Therefore, "retreatment patient" does not mean lower responder to peg-IFN + ribavirin combination therapy and all patients enrolled in this study were naïve for the combination therapy. In patients who had experienced liver biopsies (group α2a, 57 cases; group α2b, 60 cases) there were no significant intergroup differences in histopathological staging and grading (data not shown). Dose reduction of peg-IFNα and/or ribavirin, which weakens the antiviral effect, was not considered in this study, and the duration of treatment was not fixed (48 or 72 weeks). Therefore the final outcome of the treatments, SVR rates, cannot be fairly compared between the groups. However, early viral kinetics, especially the viral decline rate, may be worth evaluating because no dose reduction was done within the first 4 weeks.

Viral decline was significantly greater in group α2a compared with group α2b during the 4-12 weeks after treatment initiation (Figures 2, 3), suggesting that early viral response to peg-IFNα2a may be better than that to peg-IFNα2b. In group α2b, non-SVR patients had significantly limited viral decline during weeks 1–12 compared with SVR patients, whereas limited viral decline in non-SVR patients was found only at week 2 in group α2a (Figure 3). Accordingly, viral decline may be useful to predict SVR in group α2b but not in group α2a.

As pharmacokinetic parameters, first- and second-phase viral decline rates were compared between group α2a and group α2b. Based on the model of HCV kinetics [25,26], we devised formulae for calculating first- and second-phase viral decline rates using serum HCV RNA concentrations at baseline and week 1 and 4 after treatment initiation (Figure 1). As a result, the first-phase viral decline rate was significantly greater in group α2a, whereas the second-phase viral decline rate was comparable between the 2 groups. In some studies, ribavirin did not appear to affect first-phase viral decline, and increased second-phase viral decline when IFN response was low [27,29–32]. It has been suggested that first-phase decline reflects a dose-effect and the pharmacokinetic properties of peg-IFNs, and that the slope of the second-phase decline reflects inter-patient variability [9,33]. Peg-IFNα2a and peg-IFNα2b have different pharmacokinetics; their half-lives in plasma are approximately 77 and 40 h, respectively [34,35]. Therefore, among therapeutic factors, administered dose and half-life may be the main factors affecting the difference in first-phase viral decline rate between treatments with peg-IFNα2a *vs.* peg-IFNα2b.

In practice, it is difficult to fairly evaluate the effect of different antiviral protocols, because virological and host factors that also affect outcomes are complex. For example, novel factors such as substitution of amino acids 70 and 91 in the core region of HCV-1b [36] and genetic variation in IL28B [37–39] are associated with outcomes of antiviral therapy. In future, if these factors can be evaluated more simply and easily, more successful therapeutic protocols may be selected for individual patients as tailor-made therapy.

Conclusions

In our study population, peg-IFNα2a showed earlier viral decline than peg-IFNα2b, and the difference was particularly obvious in the first-phase viral decline, although no significant difference was shown in SVR rate between the treatments.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Alpha-fetoprotein above normal levels as a risk factor for the development of hepatocellular carcinoma in patients infected with hepatitis C virus

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Abstract
Background Noninvasive risk factors are required for predicting the development of hepatocellular carcinoma (HCC) not only in patients with cirrhosis but also in those with chronic hepatitis who are infected with hepatitis C virus (HCV).
Methods A total of 707 patients with chronic HCV infection without other risks were evaluated for the predictive value of noninvasive risk factors for HCC, including age, sex, viral load, genotype, fibrosis stage, aspartate and alanine aminotransferase levels, bilirubin, albumin, platelet count, and alpha-fetoprotein (AFP) at entry to the study, as well as interferon (IFN) therapy they received.
Results The ten-year cumulative incidence rates of HCC for patients with fibrosis stages F0/F1, F2, F3, and F4 were 2.5, 12.8, 19.3, and 55.9%, respectively. Multivariate analysis identified age ≥57 years [hazard ratio (HR) 2.026, $P = 0.004$], fibrosis stage F4 (HR 3.957, $P < 0.001$), and AFP 6–20 ng/mL (HR 1.942, $P = 0.030$) and ≥20 ng/mL (HR 3.884, $P < 0.001$), as well as the response to IFN [relative risk (RR) 0.099, $P < 0.001$], as independent risk factors for the development of HCC. The ten-year cumulative incidence rates of HCC in the patients with AFP levels of <6, 6–20, and ≥20 ng/mL at entry were 6.0, 24.6, and 47.3%, respectively.
Conclusions Not only high (>20 ng/mL), but also even slightly elevated (6–20 ng/mL) AFP levels, could serve as a risk factor for HCC to complement the fibrosis stage. In contrast, AFP levels <6 ng/mL indicate a low risk of HCC development in patients infected with HCV, irrespective of the fibrosis stage.

Keywords Alpha-fetoprotein · Hepatitis C virus · Hepatocellular carcinoma

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Introduction

Worldwide, an estimated 170 million people are persistently infected with hepatitis C virus (HCV) [1, 2], and they are at high risk of developing hepatocellular carcinoma (HCC) [1, 3–5]. Several factors have been identified that increase the risk of HCC, including, age, male gender, and alcohol intake, as well as cirrhosis and the duration of infection [3, 5]. Of these factors, the stage of liver fibrosis parallels the risk for HCV-associated HCC. The annual incidence of HCC in patients with HCV-related cirrhosis ranges from 1 to 7% [6, 7]. Although liver biopsy is the gold standard for the assessment of hepatic fibrosis [8, 9], it is too invasive a procedure to be acceptable as a routine test [10, 11]. In place of liver biopsy, the platelet count is used to estimate the degree of fibrosis [12–14], and low platelet counts have been shown to be a risk factor for the development of HCC in cirrhotic patients [13, 15, 16]. In this study, we tried to identify noninvasive markers for predicting the development of HCC in a large cohort of

patients with chronic HCV infection during a long observation period.

Patients and methods

Study design

Between January 1992 and December 2003, 832 patients were identified who were positive for both anti-HCV, by a second or third-generation enzyme-linked immunosorbent assay (ELISA), and for HCV RNA by polymerase chain reaction (PCR). These patients underwent liver biopsy guided by ultrasonography (US) at the National Nagasaki Medical Center. Of the 832 patients, 125 (15.0%) were excluded according to the following criteria: (1) positive for hepatitis B surface antigen (HBsAg) ($n = 12$); (2) heavy habitual drinking defined as an average daily consumption of >100 g ethanol ($n = 26$); (3) presence of autoimmune hepatitis (AIH), primary biliary cirrhosis, or idiopathic portal hypertension ($n = 8$); (4) positive antinuclear antibody (defined as a titer of >320×) without a diagnosis of AIH ($n = 8$); or (5) a short follow-up period (<180 days) ($n = 71$). The remaining 707 patients were analyzed retrospectively for the incidence of HCC. Their medical histories had been recorded, with the results of routine tests for blood cell counts, liver biochemical parameters, and markers for HCV infection at the time of US-guided liver biopsy at regular intervals. Complete blood cell counts and biochemical tests were performed, using automated procedures, at the clinical pathology laboratories of the National Nagasaki Medical Center. Iinformed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a-priori approval by the institution's human research committee.

Staging of hepatic fibrosis

Liver biopsy was taken by fine-needle aspiration (18G or 16G sonopsy) guided by US. Liver tissue specimens were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. They were evaluated for the stage of hepatic fibrosis by a pathologist according to the criteria of Desmet et al. [17].

HCV RNA, HCV core antigen, and HCV genotypes

HCV RNA was determined by reverse transcriptase (RT)-PCR using a commercial kit (Amplicor HCV; Roche Diagnostic Systems, Basel, Switzerland). HCV core antigen was determined using the lumispot EIKEN HCV antigen assay (Eiken Chemicals, Tokyo, Japan). HCV core antigen levels were classified as low or high with the cutoff at 1,000 fmol/L [18, 19]. Genotypes of HCV were determined by RT-PCR with genotype-specific primers (HCV RNA core genotype; Roche Diagnostics, Tokyo, Japan) [20, 21].

Interferon therapy

During the observation period, 373 of the 707 (52.8%) patients received interferon (IFN) monotherapy, pegylated (PEG)-IFN monotherapy, combination therapy with IFN and ribavirin, or PEG-IFN and ribavirin. Sustained virological response (SVR) was defined as the absence of detectable HCV RNA by the end of treatment that persisted for longer than 6 months thereafter, while failure in meeting these criteria was judged as non-SVR. There was no relapse of viremia after 6 months among SVR patients.

Diagnosis of hepatocellular carcinoma

Patients were followed up with hematological and biochemical tests at intervals of 1–12 months. Liver imaging was performed by US at 6- to 12-month intervals in most patients at fibrosis stages F0–F2, while computed tomography (CT), magnetic resonance imaging (MRI), or US was performed at 3- to 6-month intervals in patients at fibrosis stages F3 and F4. HCC was diagnosed by typical vascular patterns on CT, MRI, or angiography, or by fine-needle biopsy of space-occupying lesions detected in the liver.

Statistical analysis

Continuous variables [platelet counts, albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alpha-fetoprotein (AFP), HCV core antigen] were dichotomized with respect to the median value or clinically meaningful values in a multivariate analysis. To estimate the cumulative risk of developing HCC, the Kaplan–Meier method and the log-rank test were used. Cox proportional hazards regression analysis was performed to evaluate risk factors for HCC. Analysis was performed by Bonferroni's correction and data analysis was performed with SPSS ver. 11.0 (SPSS, Chicago, IL, USA).

Results

Characteristics at enrollment

Table 1 lists the characteristics of the 707 patients at enrollment. The median age was 57.0 years; 120 (17.0%)

Table 1 Demographic, clinical, and virological characteristics of 707 patients persistently infected with hepatitis C virus (HCV)

Age (years)	57.0 (19–79)
Male	351 (49.6%)
Observation period (years)	8.2 ± 4.4[a]
Interferon therapy	373 (52.8%)
Habitual alcohol intake	135 (19.1%)
Fibrosis stage	
F0/F1	273 (38.6%)
F2	193 (27.3%)
F3	121 (17.1%)
F4	120 (17.0%)
Platelet count ($\times 10^3/mm^3$)	156 (30–391)
Albumin (g/dL)	4.2 (2.7–5.3)
Total bilirubin (mg/dL)	0.7 (0.1–2.5)
Aspartate aminotransferase (AST; IU/L)	53 (11–422)
Alanine aminotransferase (ALT; IU/L)	82 (1–1,057)
Alpha-fetoprotein (AFP; ng/mL)	6 (1–510)
HCV core antigen	
≥1,000 fmol/L	539 (76.2%)
HCV genotype	
1b	510 (72.1%)
2a/2b	195 (27.6%)
Unknown	2 (0.3%)

Values are medians with ranges in parentheses, or means with SD in parentheses

[a] Mean ± SD

patients were diagnosed histologically with liver cirrhosis (fibrosis stage: F4) and the remaining 587 had chronic hepatitis (fibrosis stage F0, F1, F2, or F3). The median value of AFP was 6 ng/mL. The average follow-up period was 8.2 years. The patients were classified into three categories by the level of AFP; 350 patients (49.5%) had AFP levels of <6 ng/mL, 254 (35.9%) had levels between 6 and 20 ng/mL, and the remaining 103 (14.6%) had levels of ≥20 ng/mL.

IFN therapy and IFN response

Of the 120 patients with cirrhosis (fibrosis stage F4), 46 (38.3%) received IFN while the remaining 74 (61.7%) did not. The proportions of IFN-treated patients showing an SVR were 40.8% (56/137) in patients with F1; 37.6% (44/117) in those with F2; 32.8% (24/73) in those with F3; and 32.6% (15/46) in those with F4.

Risk factors for HCC

Cox regression analysis was performed on several variables, including age, sex, alcohol consumption, IFN therapy during the observation period, and biochemical as well as virological parameters. The following factors were identified as showing an increased risk for HCC by the univariate analysis: age; IFN therapy; fibrosis stage; platelet count; albumin; AST, ALT, and AFP levels; and HCV genotype (Table 2). Multivariate analysis was performed on these factors (Table 3), and the following were identified as independent risk factors: fibrosis stage (F4), AFP (6–20 and ≥20 ng/mL), age (≥57 years), and IFN therapy (SVR).

Development of HCC

During the follow-up period, HCC developed in 110 (15.6%) patients. Of the 110 patients with HCC, 58 (52.7%) were diagnosed with the disease by histological examination of biopsy-obtained or resected liver specimens. Of these 58 patients, 24 (41.3%) had hypovascular HCC.

Among the patients with HCC, only eight (7.2%) had AFP <6 ng/mL at the time of diagnosis of HCC. Figure 1 shows Kaplan–Meier estimates of the cumulative risk of HCC with respect to fibrosis stage at entry. The 10-year cumulative incidence rates of HCC for stages F0/F1, F2, F3, and F4 were 2.5, 12.8, 19.3, and 55.9%, respectively.

There were significant differences in cumulative incidence rates among the three groups of patients with different AFP levels. The 10-year cumulative risk of HCC was 6.0% in the 350 patients with AFP <6 ng/mL at the study entry, 24.6% in the 254 patients with AFP 6–20 ng/mL, and 47.3% in the 103 patients with AFP ≥20 ng/mL ($P < 0.001$) (Fig. 2). Of the 350 patients with AFP <6 ng/mL, 21 eventually developed HCC during the observation period. Fourteen of these 21 patients were ≥57 years old and 10 had fibrosis stage F3 or F4. In remarkable contrast, HCC ultimately developed in 84.5% of the patients with AFP ≥20 ng/mL.

The 10-year cumulative incidence rates of HCC were 3.1% in patients with SVR to IFN, 14.6% in patients with non-SVR, and 29.5% in the patients without IFN therapy (Fig. 3). Of the 139 patients with SVR, three (2.2%) eventually developed HCC during the observation period. These three patients had advanced fibrosis stages at the study entry (1 with F3 and 2 with F4). Figure 4 shows the cumulative incidence of HCC in the patients with different AFP levels, stratified by the fibrosis stage. In the patients with fibrosis stage F4, there were significant differences in HCC incidence between those with AFP levels of <6 and those with levels of ≥20 ng/mL.

Figure 5 shows the proportions of patients with different AFP levels stratified by the fibrosis stage. The proportion of patients with AFP <6 ng/mL decreased with the advance of fibrosis stage, and conversely, the proportion of patients with AFP ≥20 ng/mL increased with the advance of fibrosis stage. There was a strong correlation between AFP levels and the fibrosis stage.

Table 2 Factors increasing the risk for hepatocellular carcinoma (HCC), determined by univariate analysis

Features	Hazard ratio	*P* value
Age		
<57 years	1	
≥57 years	3.889	<0.001
Sex		
Female	1	
Male	1.146	0.475
Alcohol intake		
None	1	
Habitual	1.012	0.962
Interferon therapy		
None	1	
Non-SVR	0.523	0.002
SVR	0.063	<0.001
Fibrosis stage		
F0/F1	1	
F2	1.863	0.096
F3	3.985	<0.001
F4	13.045	<0.001
Platelet count		
$\geq 150 \times 10^3/mm^3$	1	
$<150 \times 10^3/mm^3$	4.644	<0.001
Albumin		
≥4.2 g/dL	1	
<4.2 g/dL	2.952	<0.001
Total bilirubin		
<0.7 mg/dL	1	
≥0.7 mg/dL	1.438	0.065
AST		
<53 IU/L	1	
≥53 IU/L	2.501	<0.001
ALT		
<82 IU/L	1	
≥82 IU/L	1.514	0.035
AFP		
<6 ng/mL	1	
6–20 ng/mL	4.628	<0.001
≥20 ng/mL	10.335	<0.001
HCV core antigen		
<1,000 fmol/L	1	
≥1,000 fmol/L	1.112	0.645
HCV genotype		
2a/2b	1	
1b	1.730	0.027

SVR sustained virological response

Table 3 Factors increasing the risk for HCC, determined by multivariate analysis

Features	Hazard ratio (95% CI)	*P* value
Fibrosis stage		
F0/F1	1	
F2	1.030 (0.471–2.253)	0.942
F3	1.682 (0.632–3.713)	0.198
F4	3.957 (1.861–8.411)	<0.001
AFP		
<6 ng/mL	1	
6–20 ng/mL	1.942 (1.066–3.538)	0.030
≥20 ng/mL	3.884 (2.014–7.433)	<0.001
Age		
<57 years	1	
≥57 years	2.026 (1.261–3.255)	0.004
Interferon therapy		
None	1	
Non-SVR	0.704 (0.453–1.094)	0.119
SVR	0.099 (0.029–0.334)	<0.001

CI confidence interval

Discussion

In the present study, four variables were identified as risk factors for HCC in patients with chronic HCV infection: fibrosis stage, AFP level, age, and IFN therapy. Previous reviews have analyzed risk factors for the development of HCC [3, 22–25]. Yoshida et al. [6] have reported that the annual incidence increases with the stage of liver fibrosis, from 0.5% in patients with stage F0 or F1 to 7.9% in patients with stage F4 (cirrhosis). In our study, the cumulative incidence of HCC increased along with the advance of fibrosis stage. AFP is used as a serological marker of HCC, and is employed in combination with US for screening HCC [3]. Several reports have shown an elevated AFP level as a risk factor for the development of HCC among patients infected with HCV [16, 25–32]. Most of the studied patients had cirrhosis that was not definitely diagnosed by clinical symptoms and ultrasonographic findings. There have been few studies on patients with chronic hepatitis C, in addition to those with cirrhosis [27]. Thus, it has been unclear whether elevated AFP levels are a risk factor for the development of HCC in patients infected with HCV. Against this background, we were prompted to analyze the utility of AFP as a risk factor for the development of HCC in patients who had been histologically diagnosed by US-guided liver biopsy. In the present study,

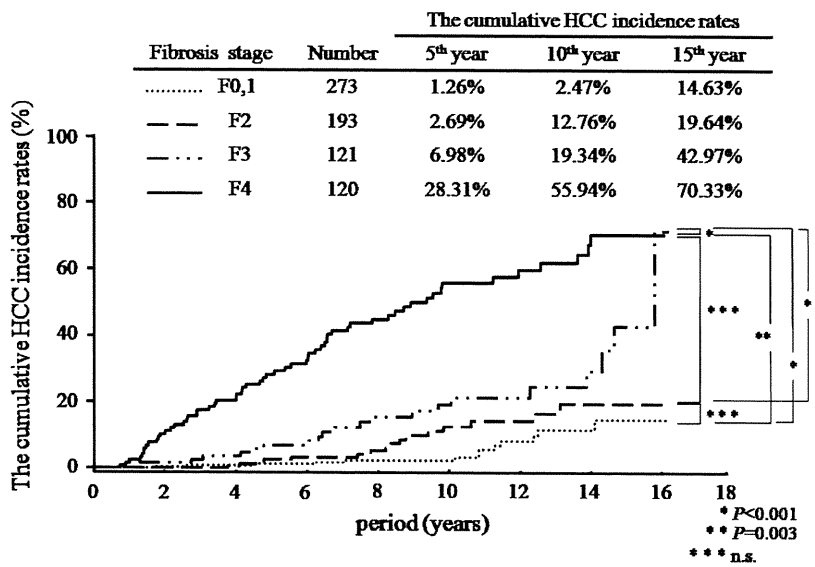

Fig. 1 Cumulative incidence of hepatocellular carcinoma (*HCC*) according to the fibrosis stage

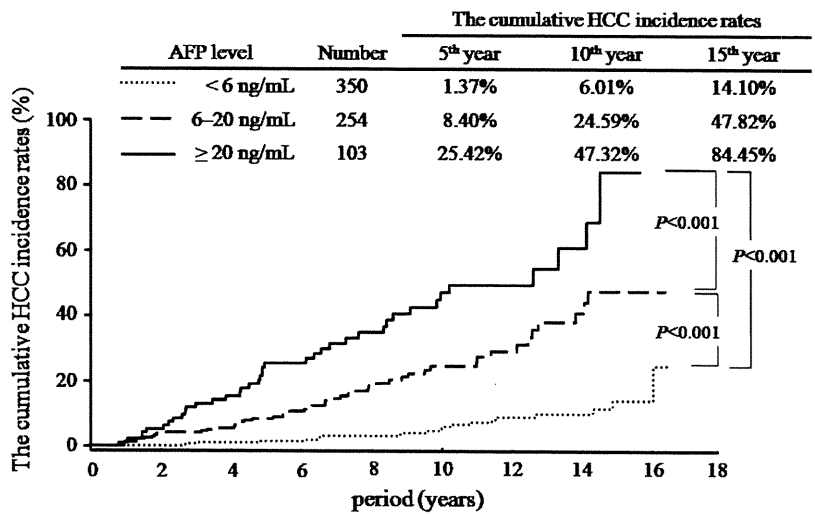

Fig. 2 Cumulative incidence of HCC according to alpha-fetoprotein (*AFP*) levels

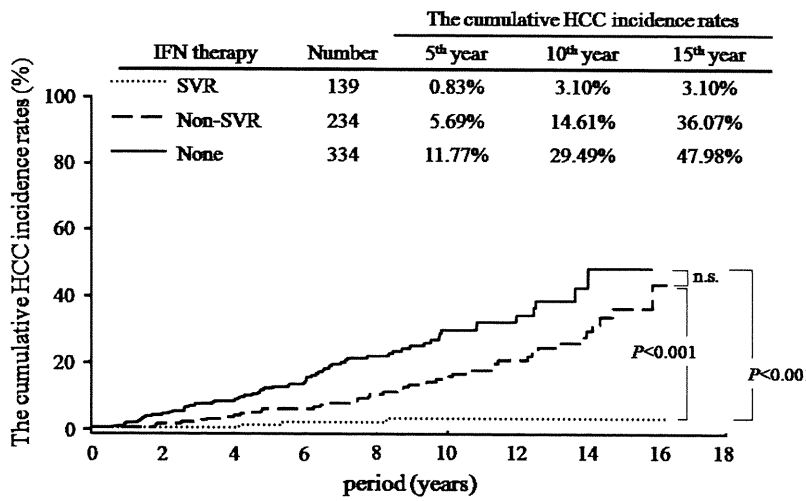

Fig. 3 Cumulative incidence of HCC according to interferon (*IFN*) therapy. *SVR* Sustained virological response

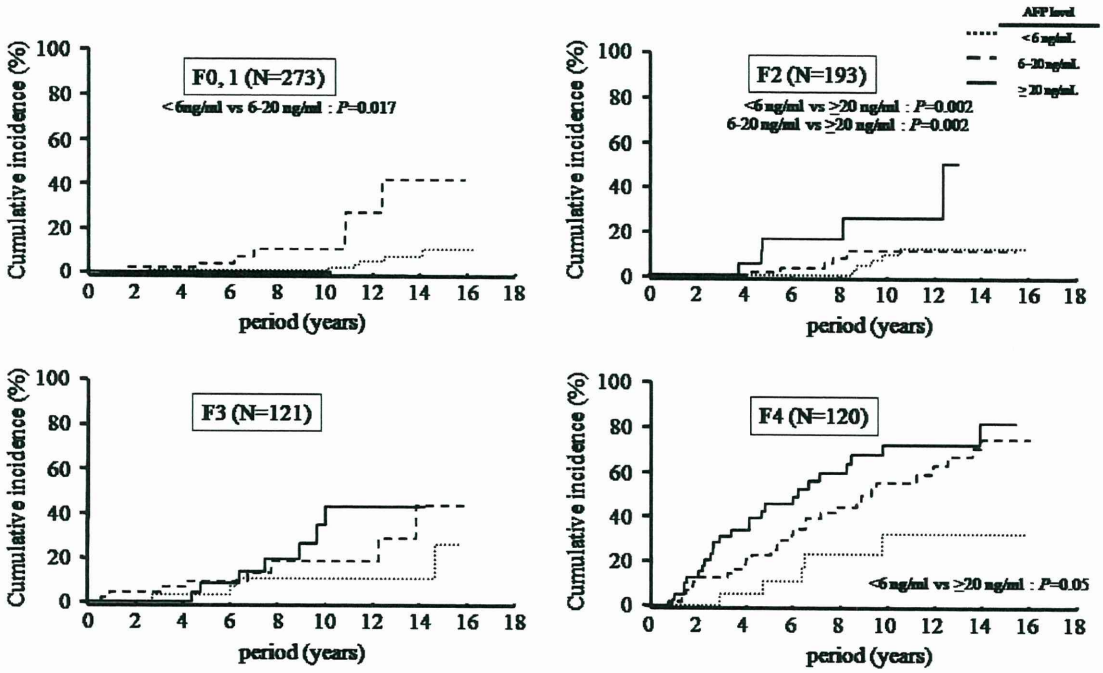

Fig. 4 Cumulative incidence of HCC according to AFP levels, stratified by the fibrosis stage

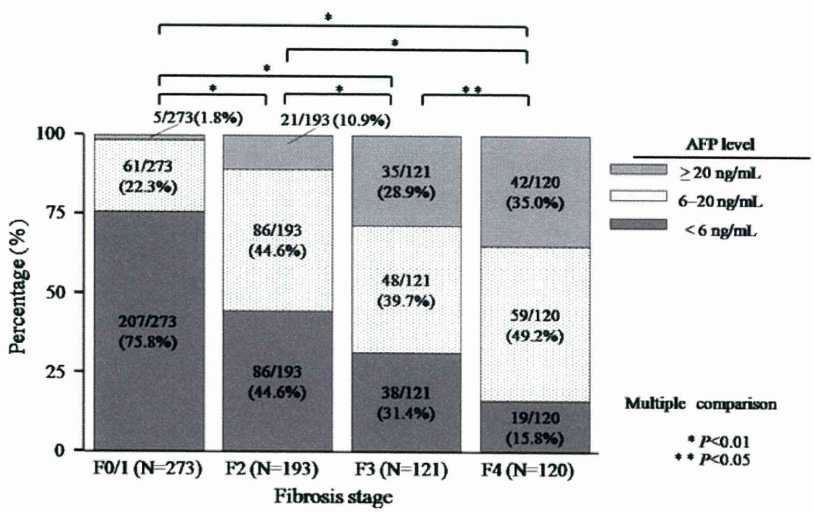

Fig. 5 Proportions of patients with three different AFP levels (<6 ng/mL, 6–20 ng/mL, and ≥20 ng/mL) at different fibrosis stages

among patients infected with HCV, including not only those with cirrhosis but also those with chronic hepatitis, we found AFP levels to be a dependable risk factor for HCC, in addition to the fibrosis stage. Of particular note, not only the patients with high AFP levels (≥20 ng/mL) but also those with even slightly elevated AFP levels (between 6 and 20 ng/mL) had increased risks for the development of HCC. In the patients in this study, the median AFP level was 6 ng/mL. It deviated slightly from serum levels of AFP in healthy adults that have been reported to range from 0.1 to 5.8 ng/mL [33]. Hence, we performed analyses by setting various AFP cutoff levels for evaluating their performance as risk factors. However, there were no significant differences in the analysis with the use of AFP cutoff levels exceeding 7 ng/mL. On the basis of these observations, an AFP cutoff level of 6 ng/mL was adopted in this study. In previous reports, AFP levels were associated with advanced fibrosis stage in patients infected with HCV in the absence of HCC [34–38]. In the present study, AFP levels were elevated in parallel with advanced fibrosis stages and correlated well with the fibrosis stage. As the patients with even slightly elevated AFP levels, between 6 and 20 ng/mL, had moderately advanced liver fibrosis stages, these AFP levels could

indicate an elevated risk for HCC in patients with chronic HCV infection.

Hu et al. [36] found that an AFP level of 15.0 mg/mL could detect severe fibrosis with a sensitivity of 22.8% and specificity of 94.5%. Moreover, they reported, during observation for 6 months of patients with chronic hepatitis C, that AFP levels stayed within the normal range (<10 ng/mL) in 60%, were persistently elevated in 24%, and fluctuated in the remaining 15%. By multivariate analysis, they identified AST, INR, and fibrosis as risk factors for AFP levels of >10 ng/mL. In view of the correlation between AFP levels and fibrosis stages, the AFP level at the time of liver biopsy was taken into account in the analysis in the present study; ALT levels are reported to be persistently elevated in the majority (60%) of patients with chronic hepatitis C.

Liver biopsy is the gold standard for assessing hepatic fibrosis [8, 9]. However, the needle liver biopsy has a sampling error and is too invasive as a routine procedure [10, 11]. Therefore, AFP levels may be used as a noninvasive and predictive marker in place of the fibrosis stage. The platelet count is known to reflect the severity of chronic hepatitis C [12, 13], and is used to estimate the degree of fibrosis without resort to liver biopsy [12–14]. Previous reports have shown low platelet counts to represent a risk factor for HCC in cirrhotic patients [13, 15, 16]. Matsumura et al. [13] reported that age and serum platelet count were significant risk factors for the development of HCC, and as such, they were a major clinico-laboratory means of evaluating the fibrosis stage. In the present study, however, the platelet count was not an independent risk factor for HCC development. When Cox regression analysis was performed on variables other than the fibrosis stage, platelet count and serum albumin levels were identified as independent risk factors for the development of HCC (data not shown).

IFN has been used to treat patients with HCV infection. Failure to achieve an SVR to IFN-based therapies, and preexisting advanced hepatic fibrosis and/or cirrhosis, are major predictors of HCC [6, 23, 25, 39, 40]. In the present study, SVR emerged as an independent risk factor for the development of HCC, while non-SVR was not. However, the cumulative incidence rate of HCC in patients with non-SVR was lower than that in those without IFN therapy. These results suggest that the use of IFN in patients with HCV-related liver disease may be beneficial in preventing the development of HCC. Several Japanese cohort studies have demonstrated that IFN therapy reduces the incidence of HCC, not only in sustained virological responders but also in transient responders who have failed to eliminate HCV [6, 41–45]. In cirrhotic patients, Nishiguchi et al. [39] reported that the relative risk of patients with IFN-alpha treatment developing HCC was 0.067 in comparison with the control group. In contrast, Valla et al. [46] could not prove any significant benefit for the prevention of HCC between patients with and without IFN treatment. Camma et al. [47] suggested a slight preventive effect of IFN on HCC development in patients with HCV-related cirrhosis. Shiffman et al. [48] have reported that continuous IFN therapy led to a decline in hepatic fibrosis despite the persistence of viremia. In addition, there are case reports that IFN therapy reduced AFP levels in virological nonresponders [49]. Murashima et al. [50] showed that IFN therapy, but not Strong Neo-Minophagen C (SNMC) (Glycyrrhizin, Tokyo, Japan), universally reduced basic AFP levels. In an in vitro study of the effects of IFN on an HCC cell line, IFN exhibited antitumor effects [51]. Taken together, these findings suggest that AFP levels may be useful for predicting the development of HCC during IFN-based treatments, including long-term low-dose IFN therapy.

There have been several reports on the relationship between chronological trends in platelet counts, AST or AFP levels, and the development of HCC [11, 26, 27, 52–54]. Tarao et al. [52, 53] showed that in patients with HCV-related cirrhosis, those with persistently high serum ALT levels had a high risk of developing HCC and multicentric carcinogenesis, whereas those with persistently low ALT levels faced a very low risk. Likewise, the dynamics of AFP levels in patients with chronic HCV infection may be useful to estimate the risk of developing HCC. Recently, Bruce et al. [32] found serial measurements of AFP helpful in identifying persons with advanced fibrosis. They used an AFP level of 8 ng/mL, the test manufacturer's upper limit of normal, as the evaluation of the risk of development of HCC. It is not certain whether or not AFP would be a risk factor of HCC development in patients with chronic liver disease of etiologies other than persistent HCV infection. Velazquez et al. [55] reported that an AFP level of >5 ng/mL at study entry was associated with the development of HCC in their univariate analysis but not in their multivariate analysis. They speculated that this could have been because the main causative factor of liver cirrhosis in their series was alcohol. Taken together, the findings of various studies suggest that the baseline AFP level may be more reliable as a predictive factor for the development of HCC in patients with HCV-related liver disease than in those with liver disease of other etiologies.

In conclusion, AFP is a noninvasive predictive marker for the development of HCC in patients infected with HCV. The present study indicates that not only high AFP levels (≥20 ng/mL) but also slightly elevated AFP levels, between 6 and 20 ng/mL, could indicate substantial risks for the development of HCC, complementing the fibrosis stage. In contrast, AFP levels of <6 ng/mL indicate a low risk of HCC development, irrespective of the liver fibrosis stage. IFN therapy significantly reduces the risk of the

development of HCC, especially in patients with an SVR to the therapy.

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Dedifferentiation of Human Primary Thyrocytes into Multilineage Progenitor Cells without Gene Introduction

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Abstract

While identification and isolation of adult stem cells have potentially important implications, recent reports regarding dedifferentiation/reprogramming from differentiated cells have provided another clue to gain insight into source of tissue stem/progenitor cells. In this study, we developed a novel culture system to obtain dedifferentiated progenitor cells from normal human thyroid tissues. After enzymatic digestion, primary thyrocytes, expressing thyroglobulin, vimentin and cytokeratin-18, were cultured in a serum-free medium called SAGM. Although the vast majority of cells died, a small proportion (~0.5%) survived and proliferated. During initial cell expansion, thyroglobulin/cytokeratin-18 expression was gradually declined in the proliferating cells. Moreover, sorted cells expressing thyroid peroxidase gave rise to proliferating clones in SAGM. These data suggest that those cells are derived from thyroid follicular cells or at least thyroid-committed cells. The SAGM-grown cells did not express any thyroid-specific genes. However, after four-week incubation with FBS and TSH, cytokeratin-18, thyroglobulin, TSH receptor, PAX8 and TTF1 expressions re-emerged. Moreover, surprisingly, the cells were capable of differentiating into neuronal or adipogenic lineage depending on differentiating conditions. In summary, we have developed a novel system to generate multilineage progenitor cells from normal human thyroid tissues. This seems to be achieved by dedifferentiation of thyroid follicular cells. The presently described culture system may be useful for regenerative medicine, but the primary importance will be as a tool to elucidate the mechanisms of thyroid diseases.

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Introduction

Adult stem cells are rare, premature cells capable of self-renewal and generating distinct differentiated cell types within a tissue. There is no doubt that identification and isolation of stem cells have potentially important implications not only for regenerative medicine but for understanding of pathogenesis of a variety of diseases.

The existence of stem cells in adult human thyroid tissue has been suggested by several studies. Based on immunohistochemical analyses, two groups proposed that p63-positive cells residing in solid cell nests may be undifferentiated stem cells and undergo follicular maturation [1,2,3,4]. Thomas et al. found that a small subset of cells in nodular goiters express a stem cell marker *Oct-4* and endodermal markers *GATA-4* and *HNF4α* using RT-PCR, immunohistochemistry and flow cytometry; however, no functional characterization was performed [5].

The subsequent work by the same group (Lan et al.) reported that possible stem cells were isolated from nodular goiters using a sphere formation approach in a serum-free medium containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), and some functional studies were performed [6]. This method has been frequently used to isolate stem cells from several other tissues. The obtained sphere cells did not express thyroid specific genes such as *thyroglobulin (TG)* and *TSH receptor (TSH-R)*, but their expressions emerged in monolayer culture stimulated by serum and TSH. However, the growth potential of the cells was very limited: the proliferation stopped after 4–5 days of culture.

More recently, Fierabracci et al. have also reported the identification of stem/progenitor cells in normal human thyroid tissues [7]. They also used similar approach with serum-free/EGF/bFGF medium and sphere formation. The isolated cells were probably not fully undifferentiated or not homogeneous, since TG or thyroid peroxidase (TPO) expression was in part

detected. After stimulation with serum, the cells formed follicle-like structure in collagen gel and produced T_4. Surprisingly, some isolated cells underwent multilineage differentiation into neurogenic and adipogenic lineages.

So far, two above-mentioned studies described isolation and functional characterization of possible stem cells from human thyroid tissues (including goiters). However, there are still some uncertainties as for origin and homogeneity of the cells. In addition, Lan et al. used cells released by enzymatic digestion, whereas Fierabracci et al. used residual thyroid fragments, presumably containing cells tightly bound to collagen fibers, regardless of using very similar medium. Therefore, the isolated cells by Lan et al. may be different from those by Fierabracci et al. We tried both methods but could not reproduce the sphere formation in our hands (unpublished data). Thus, the origin and procedure of isolation of thyroid stem cells still remain obscure.

Recent reports have provided another clue to gain insight into source of primitive stem/progenitor cells. In 2007, induced pluripotent stem (iPS) cells were established from adult human fibroblasts by direct reprogramming with defined factors [8]. Recently, not only reprogramming (generating pluripotent stem cells) but also generating tissue stem/progenitor cells (i.e. dedifferentiation or partial reprogramming) has also been reported. Mani et al. have demonstrated that differentiated mammary epithelial cells can be converted into mammary tissue stem cells by introducing genes related to epithelial-mesenchymal transition (EMT) [9]. Moreover, cancer stem cells (tumor-initiating cells) have also been generated from cancer cells [10] or even from normal epithelial cells [11]. These reports suggest that tissue stem/progenitor cells could be generated from mature and differentiated cells.

In the present study, we present a novel culture system in which progenitor cells can be emerged and propagated from normal human thyroid follicular cells by dedifferentiation. We also demonstrate that the cells possess multilineage differentiation potential.

Materials and Methods

Cell culture and reagents

We used anaplastic thyroid carcinoma cell line: FRO; papillary thyroid carcinoma cell lines: TPC-1 and KTC-1; follicular thyroid carcinoma cell line: WRO. All these cells were of human origin. FRO and WRO were kindly provided by Dr G. Juillard (University of California, Los Angeles, CA). TPC-1 and KTC-1 were kindly provided by Dr Sato (Kanazawa University, Kanazawa, Japan) and Dr Kurebayashi (Kawasaki Medical School, Kurashiki, Japan), respectively. All the cells were maintained in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and penicillin/streptomycin (Sigma, St. Louis, MO) at 37°C in a humidified atmosphere with 5% CO_2. Primary human thyroid cells (PT) were isolated from thyroid tissues obtained during subtotal thyroidectomy in patients with Graves' disease as described previously [12] and cultured overnight in DMEM:F12 medium (1:2) supplemented with 3.3% FBS and penicillin/streptomycin (PT medium). All experiments were performed after obtaining approval by the Ethical Committee of Nagasaki University Graduate School of Biomedical Sciences. Written informed consent was obtained from each individual. SAGM was purchased from Lonza (Basel, Switzerland). For neurogenic and adipogenic differentiation, Human Neural Stem Cell Functional Identification Kit (R&D Systems, Minneapolis, MN) and StemPro Adipogenesis Differentiation Kit (Invitrogen) were used, respectively. Bovine TSH (bTSH) was purchased from Sigma. Oil-red-O staining was performed using Lipid Assay kit (Primary Cell Co., Sapporo, Japan).

Immunofluorescence

Cells cultured on coverslips were fixed and immunolabeled as previously described [13]. The following primary antibodies were used: anti-phosphorylated H2AX (Ser139) (mouse clone JBW301, Millipore, Billerica, MA), anti-cytokeratin-18 (Clone DC 10, Dako Cytomation, Glostrup, Denmark), anti-vimentin (Clone V9, Dako Cytomation), anti-STRO-1 (Clone STRO-1, R&D Systems) and anti-TG (mouse clone B34.1, GeneTex, Irvine, CA). Anti-β-III-tubulin antibody was included in Human Neural Stem Cell Functional Identification Kit (R&D Systems). After labeling with secondary antibodies conjugated with Alexa 488 or 594 (Invitrogen), images were acquired using a fluorescence microscopy DM6000B (Leica Microsystems, Tokyo, Japan).

Senescence-associated β-galactosidase (SA-β-gal) staining

SA-β-gal staining was carried out as described by Dimri et al. [14]. Briefly, cells were fixed with 2% paraformaldehyde solution containing 0.2% glutaraldehyde for 5 min at room temperature. After fixation, the cells were then incubated with stain solution (40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$) containing 1 mg/ml X-gal at 37°C for overnight.

Fluorescence-activated cell sorting

Isolated PT were cultured overnight in PT medium supplemented with 10 mIU/ml bTSH. On the next day, PT were collected and incubated with anti-STRO-1 or anti-TPO (Clone MoAb47, Dako Cytomation) antibody. After labeling with secondary antibody conjugated with Alexa 488 and 7-AAD (BD Biosciences, Franklin Lakes, NJ), the cells were analyzed and sorted using a FACS Vantage SE (BD Biosciences).

Real-time RT-PCR

Total RNA was extracted using ISOGEN reagent (Nippon gene, Tokyo, Japan) and reverse transcribed using SuperScript III First-Strand Synthesis SuperMix (Invitrogen) with random hexamers. The following PCR amplifications were performed using QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) for TTF1 and SYBR Premix Ex Taq Perfect Real Time kit (TaKaRa Bio, Ohtsu, Japan) for other genes in a Thermal Cycler Dice Real Time System (TaKaRa Bio). The cycle threshold value, which was determined using second derivative, was used to calculate the normalized expression of the indicated genes using Q-Gene software [15], using ribosomal RNA 18S as a reference. The following primer pairs were used: 18S, 5′-GTAACCCGTTGAACCCCATT-3′ and 5′-CCATCCAATCGGTAGTAGCG-3′; TG, 5′-CTGGTGTGTCATGGACAGCGGAGAA-3′ and 5′-CCCGAGATTGTCTCACACAGGAT-3′; TSH-R 5′-CTATAGATGTGACTCTGCAGCAGCT-3′ and 5′-GAGGGCATCAGGGTCTATGTAAGT-3′; PAX8 5′-CCCCCTACTCCTCCTACAGC-3′ and 5′-ACTGTCCCCATGGCAACTAC-3′; TTF1 5′-CCATGAGGAACAGCGCCTC-3′ and 5′-CTCACG TCCCCCAGCGA-3′.

Microarray analysis

Total RNAs were extracted from PT from three different samples (PT0808, PT0811 and PT0812) on the next day of initial plating and corresponding SAGM-grown lines at two weeks after

plating using ISOGEN reagent (Nippon gene) and subjected to Affymetrix GeneChip Human Genome U133 Plus 2.0 microarray analysis service (Bio Matrix Research, Nagareyama, Japan). The array contained 54,675 probes for mRNAs. GeneSpring Software (Agilent Technologies, Santa Clara, CA) was used for data analyses. The data were deposited in NCBI GEO site (accession number: GSE24553).

Results

Isolation of proliferative cells from normal human thyroid tissues

In an attempt to isolate primitive human thyroid epithelial cells, we tried several commercially available media containing growth factors but not serum which is generally thought to enforce differentiation program. Those were basically developed for growing human primary cells or tissue stem cells. Among them, we found that SAGM was the most suitable for our purpose, which was developed for growing human primary small airway epithelial cells.

After enzymatic digestion of thyroid tissues, primary thyrocytes (PT) were plated in PT medium containing FBS which facilitated attachment of the cells. On the next day, the PT medium was replaced by SAGM (Figure 1A; a). Within 7–10 days culture, the vast majority of the cells died (Figure 1A; b). This was probably due to induction of apoptosis, which was confirmed by nuclear fragmentation and γ-H2AX staining (massive DNA double strand breaks, Figure 1A; c) [16]. By contrast, clonal expansion of a small proportion became evident by 7–10 days culture, and they grew continuously (Figure 1A; d–f). These cells showed mesenchymal cell-like morphology (Figure 1A; e, f). Doubling time of the cells was approximately 24–36 hours during first 1–2 months. Time-lapse analysis looking at cell cycle length demonstrated that some of them showed asymmetric division in culture (Figure S1). The SAGM-grown cells had 46 chromosomes with no apparent gross rearrangement by Giemsa staining. Growth rate of the cells gradually declined and eventually became flat by approximately 3–4 months. At this point, the cells were positive for SA-β-gal staining, indicating that they became senescent (Figure 1B).

We used more than 10 tissues from Graves' patients and successfully obtained proliferative cells from all of them. On average, approximately 40–50 cells were estimated to have clonogenic potential among 10,000 PT (Figure 1C).

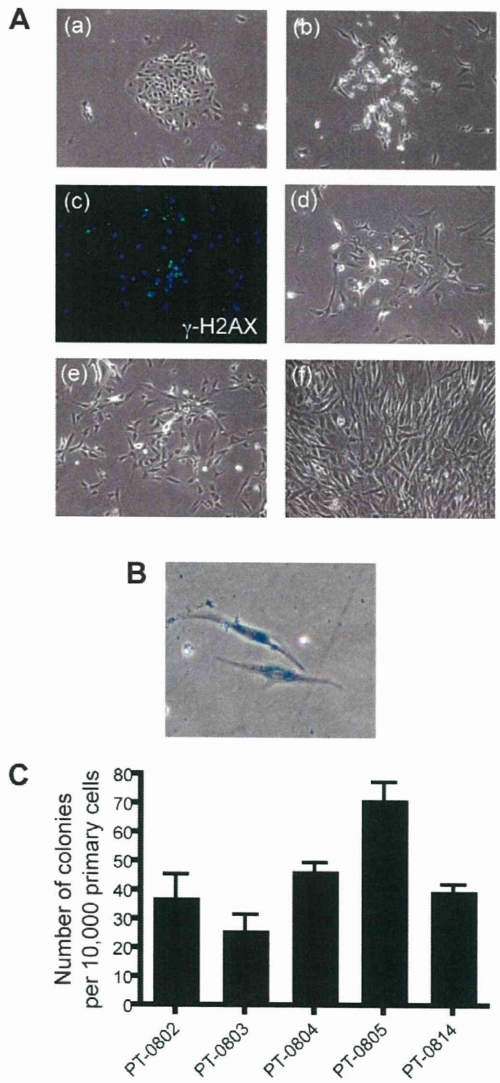

Figure 1. Isolation of proliferative cells from normal human thyroid tissue. A, (a) PT on the next day of plating. (b) A cluster of dead cells within 7–10 days. (c) γ-H2AX staining of dead cells. (d–f) Expansion of cells after 7–10 days. B, After cultured for three months, the cells showed positive for SA-β-gal staining. C, Clonogenic ability of the primary cells incubated with SAGM. Each bar indicates the mean and SD of three dishes.
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Characteristic expression of intermediate filaments in thyroid cells

To characterize the SAGM-grown cells, we investigated expression of intermediate filaments because their expression pattern depends on type of cell lineage. First of all, we checked the expression in PT. As shown in Figure 2A, cytokeratin-18, generally found in single layer epithelial tissues, was abundantly expressed in PT. Expression of vimentin, which is usually found in non-epithelial type of cells such as fibroblasts and endothelial cells, was also observed in PT (Figure 2A). TG expression was confirmed, ensuring that these cells are *bona fide* thyroid follicular cells (Figure 2A). Next, we checked the cytokeratin-18 and vimentin expression in thyroid cancer cell lines. FRO, TPC-1 and WRO expressed both filaments as well as PT (data not shown). These data suggest that both cytokeratin-18 and vimentin are expressed in normal thyroid and thyroid cancer cells. We then examined the SAGM-grown cells and found that only vimentin but not cytokeratin-18 was expressed in these cells (Figure 2B).

Origin of the SAGM-grown cells

The above observations prompted us to explore the origin of the SAGM-grown cells because all examined thyroid cells even anaplastic cancer cell line expressed cytokeratin-18. Since vimentin is used as a marker of mesenchymal cells, we next used a mesenchymal stem cell marker STRO-1. Three days after plating (no dead cells at this time point), a small number of cells were positive for STRO-1, and these cells were negative for cytokeratin-18 (Figure 2C, arrowheads). We found that all cytokeratin-18-negative cells were STRO-1/vimentin-positive. This was confirmed by analyzing about 4,500 cells by fluorescence microscopy (Table 1). Vimentin was expressed in all attached cells (Table 1). These data indicate that there were two types of

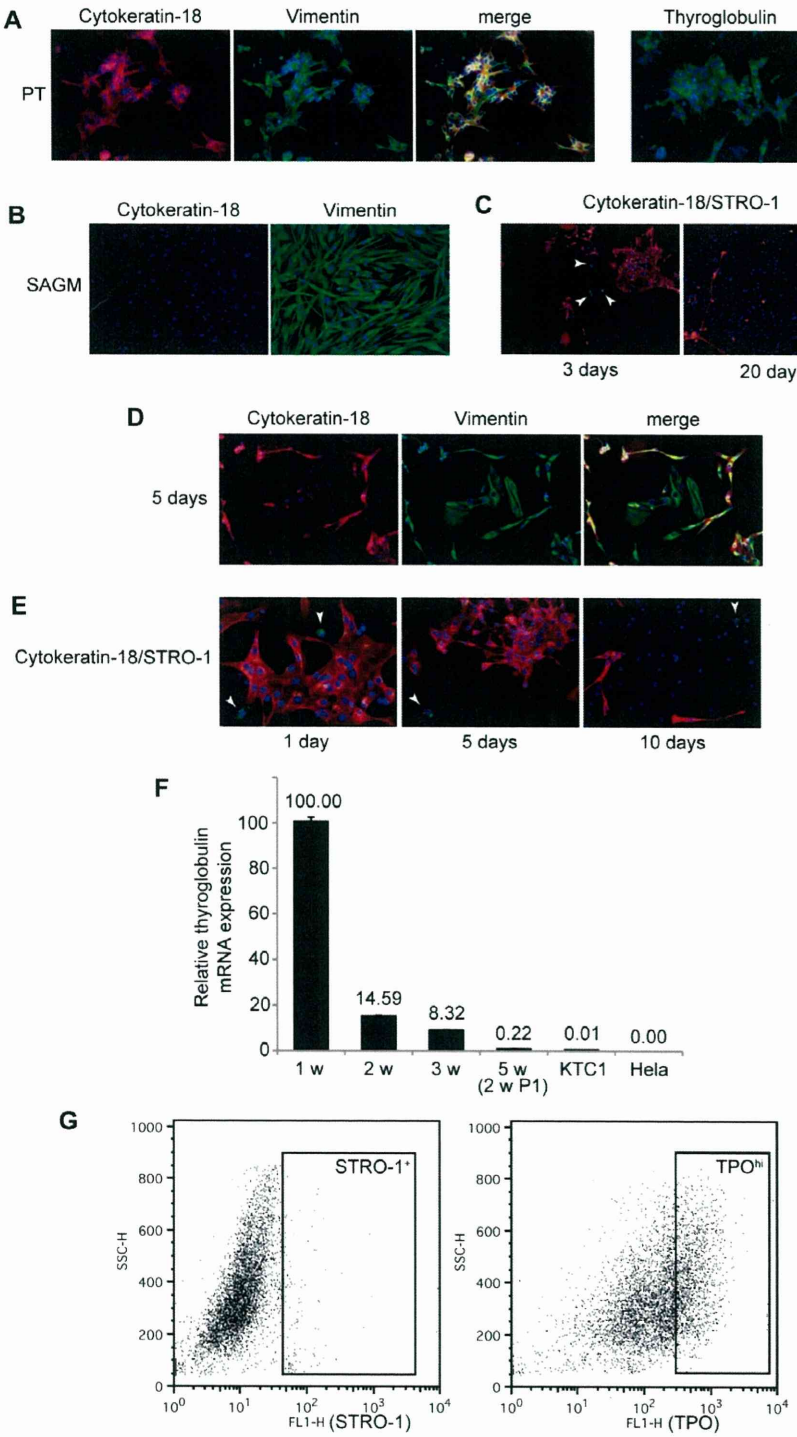

Figure 2. Origin of the SAGM-grown cells. A, On the next day after plating, PT were fixed and followed by immunofluorescence staining using following primary antibodies: cytokeratin-18 (red), vimentin (green) and TG (green). B, After grown for three weeks, the SAGM-grown cells were fixed and stained for cytokeratin-18 (red) and vimentin (green). C and E, Cells were fixed and then stained for STRO-1 (green) and cytokeratin-18 (red) on indicated days after plating. Arrowheads indicate STRO-1-positive cells. D, Five days after plating, cells were fixed and stained for cytokeratin-18 (red) and vimentin (green). At the center of each image, cell cluster just starting expansion is shown. F, At indicated time points, total RNA was extracted and then subjected to qRT-PCR for *TG*. Each bar indicates the mean and SD of the data collected in triplicate. 2 w P1: two weeks after first passage (the cells were subcultured at three weeks). RNAs from KTC-1 and Hela cells were also used as controls. Similar results were obtained in three different samples. G, On the next day after plating, PT were stained with anti-STRO-1 or anti-TPO antibody and subjected to FACS. Each box means sorting gate for STRO-1-positive or TPO^{hi} cells.
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Table 1. Expression of lineage-specific markers in primary thyrocytes.

Cells	Total count	Cyt^+	Vim^+	Cyt^+/Vim^+	$Vim^+/STRO\text{-}1^+$
PT-0808	4507	4472 (99.2)	4507 (100)	4472 (99.2)	35 (0.8)
PT-0811	4856	4809 (99.0)	4856 (100)	4472 (99.0)	47 (1.0)
PT-0818	4728	4686 (99.1)	4728 (100)	4686 (99.1)	42 (0.9)
PT-0901	4413	4376 (99.2)	4413 (100)	4376 (99.2)	37 (0.8)

No. of cells (%).
Determined by immunofluorescence.
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attached cells in the culture at this time point: cytokeratin-18/vimentin double-positive cells and vimentin/STRO-1 double-positive cells. By contrast, the SAGM-grown cells (20 days after plating) did not express STRO-1 (Figure 2C).

Next, we carefully investigated the process of the beginning of proliferation by fixing cells every two days. We found clusters with 5–10 cells just starting expansion 5–7 days after plating (Figure 2D). As expected, vimentin was expressed in these cells, while cytokeratin-18 was only weakly expressed in the same cells (Figure 2D), suggesting that the proliferating cells were losing cytokeratin-18 expression. In contrast, at the same time point, vimentin/STRO-1-positive and cytokeratin-18-negative cells did not proliferate at all (Figure 2E, arrowheads; Table S1).

We also measured relative expression of *TG* mRNA by real-time quantitative RT-PCR (qRT-PCR). At one week, *TG* expression was due to residual differentiated thyroid follicular cells (Figure 2F). The amount of *TG* mRNA gradually decreased; however, the SAGM-grown cells after three-week culture still expressed low level of *TG* mRNA, which was still higher than that in KTC-1 cells (Figure 2F). KTC-1 cells show relatively higher *PAX-8* and *TTF-1* transcripts among thyroid cancer cell lines [17].

To further confirm the origin of the proliferating cells, we next performed selective cell culture in SAGM after fluorescence-activated cell sorting (FACS) using anti-STRO-1 (for sorting STRO-1-positive cells) and anti-TPO (for sorting thyroid follicular cells) antibodies (Figure 2G). The percentages of TPO-positive cells were 70–90% depending on samples, and we sorted top 40% (TPO^{hi} cells) for subsequent cultures. As expected, STRO-1-positive (approximately 1%) cells did not grow at all in SAGM, whereas TPO^{hi} cells gave rise to proliferating colonies (Table S2).

Taken together, these results suggest that the SAGM-grown cells were derived from thyroid follicular cells or at least thyroid-committed cells.

Differentiation of the SAGM-grown cells

The SAGM-grown cells were incubated with PT medium supplemented with 10 mIU/ml bTSH, and the expression of cytokeratin-18 and TG was examined at different time points. The growth of the cells stopped in this medium. As shown in Figure 3A, the expression of cytokeratin-18 was gradually increased (Figure 3A). TG expression was also evident after 30 days stimulation (Figure 3A). We also checked mRNA expression of *TSH-R*, *TG*, *PAX8* and *TTF-1* by qRT-PCR. Although the levels of induction varied depending on the samples, all of the messages were gradually increased (Figure 3B). Interestingly, serum stimulation alone (without bTSH) up-regulated *TSH-R* expression, whereas *TG* induction needed both serum and bTSH stimulation (Figure 3B). These data indicate that the SAGM-grown cells were differentiated into thyroid follicular lineage. We next explored the differentiation potential of the cells into other lineages. Surprisingly, after incubation with the neurogenic differentiating medium for four weeks, the SAGM-grown cells expressed β-III-tubulin, which is a microtubule element of the tubulin family found almost exclusively in neurons (Figure 3C). Moreover, after four-week-treatment with the adipogenic differentiating medium, many lipid droplets were formed, and they were all positive for oil-red-O staining (Figure 3C). We measured the proportion of differentiation marker-positive cells in each differentiating condition. In thyroid differentiation, most of cells (>90%) were positive for TG, while β-III-tubulin-positive and oil-red-O-positive cells varied (48–87%) presumably depending on conditions (Table 2). These data suggest that the SAGM-grown cells have multipotent (at least dipotent) differentiation potential.

Gene expression profile of the SAGM-grown cells

To perform a comprehensive analysis of differential gene expressions between PT and SAGM-grown cells, we used oligonucleotide-based DNA microarrays, GeneChip Human Genome U133 Plus 2.0 array (Affmetrix). This array system utilizes flag score (present, marginal and absent) calculated by the difference in signals between perfect match (PM) and mismatch (MM) probes. Probes with absent call likely represent undetectably low expression (but not always), and therefore, the fold-change is not accurate. Of 54,675 probe-sets, 27,535, 26,800 and 26,929 were scored as "present call" (neither marginal nor absent) in both PT and SAGM-grown cells of PT0808, PT0811 and PT0812, respectively. The tree view of hierarchical clustering using probes with present call indicated distinct patterns in gene expressions between PT and SAGM-grown cells (Figure 4A).

We next checked the fold-change of interested genes (Table 3). Probes with absent call in either PT or SAGM sample are also included because it is still possible to estimate the significant change even though its fold-change is not reliable. Stem cell marker *ABCG2*, *Oct-4* and *CD133* were not expressed in the SAGM-grown cells. Thyroid-specific genes such as *TG*, *TSH-R*, *PAX8*, *TTF1* and *TPO* seemed to be highly suppressed, which was validated by qRT-PCR (Figure 4B). Among other tissue stem cell markers, *CD106*, *CD105* and *CD90*, which are marker for mesenchymal and/or hematopoietic stem cell, were up-regulated. *E-cadherin*, whose loss is correlated with induction of EMT, was highly down-regulated.

Next, we selected 663 genes that were significantly (t-test, $p<0.05$) and >2.0-fold increased in all the SAGM-grown cells compared to corresponding PT. Likewise, 649 genes were selected as down-regulated genes (<0.5-fold). Probes with absent call in either PT or SAGM sample are excluded. To get insight into the biological significance, Gene Ontology (GO) enrichment analysis was performed to identify specifically regulated biological processes using the above selected genes. Sixteen and four GO processes were significantly impacted ($p<0.1$) in the up- and down-regulated gene sets, respectively (Table 4). Interestingly, some particular processes were impacted. Processes related to embryo implantation, endoplasmic reticulum, sterol/steroid, cell cycle and oxidoreductase were up-regulated, and those related to protein binding and cell adhesion were down-regulated (Table 4).

Discussion

In the present study, we have developed a novel system to trigger dedifferentiation of normal human thyroid follicular cells. We used a commercially available medium which allowed universal and reproducible procedure. We investigated the origin

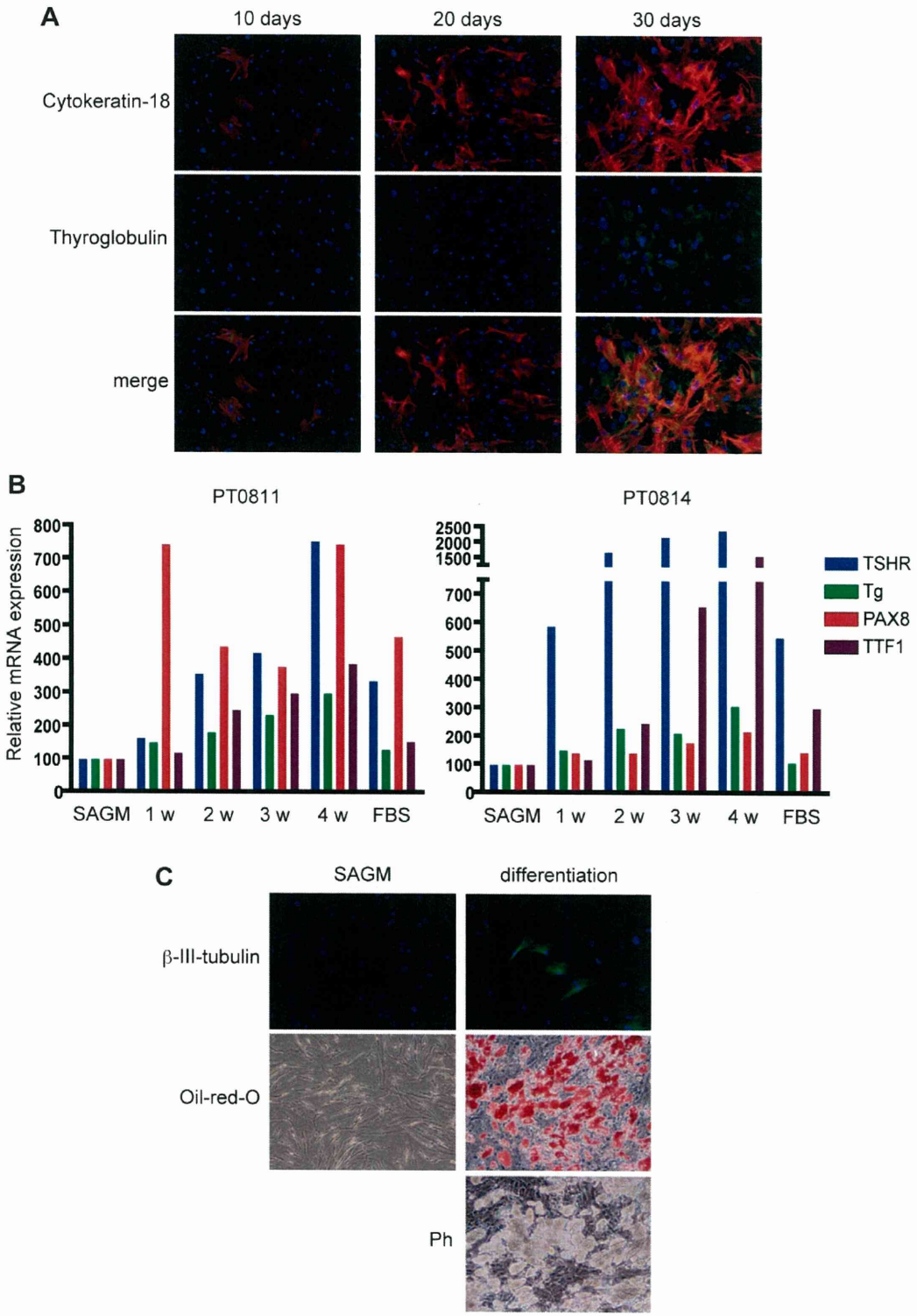

Figure 3. Differentiation of the SAGM-grown cells. A, The SAGM-grown cells were incubated with PT medium supplemented with bTSH for indicated times. The cells were then fixed and stained for cytokeratin-18 (red) and TG (green). B, At indicated time points, total RNA was extracted and subjected to qRT-PCR for indicated gene expressions. Each bar indicates the mean of the data collected in duplicate. C, The SAGM-grown cells were incubated with neurogenic differentiation medium or adipogenic differentiation medium for four weeks. The cells were fixed and stained for β-III-tubulin (upper, immunofluorescence) or lipid (middle, oil-red-O staining). Ph: phase contrast image of cells with lipid droplets. The data are representative of three independent experiments.
doi:10.1371/journal.pone.0019354.g003

Table 2. Percentage of differentiated marker-positive cells in each differentiating condition.

Cells	Tg^+	β-III-tub^+	Oil-red-O^+
PT-0808	96.8	67.1	87.0
PT-0902	93.9	55.0	48.6

% of positive cells.
doi:10.1371/journal.pone.0019354.t002

of the cells by analyzing expressions of STRO-1 and two intermediate filaments, cytokeratin-18 and vimentin. In our system, there were only two types of attached cells in terms of expression pattern of the above-mentioned markers after initial plating: cytokeratin-18/vimentin double-positive cells and STRO-1/vimentin double-positive cells. Thyroid follicular cells coexpress cytokeratin-18 and vimentin, which is consistent with earlier studies [18,19,20]. Cytokeratin-18/vimentin-positive cells may also contain a small number of endothelial cells since several papers demonstrated expression of cytokeratin in microvascular endothelial cells in different tissues [21,22,23,24,25]. STRO-1/vimentin-positive cells are perhaps composed of premature mesenchymal cells. The SAGM-grown cells were propagated from thyroid follicular cells (or at least thyroid-committed cells) because: (1) the SAGM-grown cells were negative for STRO-1; (2) the cells lost cytokeratin-18 expression during expansion; (3) STRO-1-positive cells did not proliferate; (4) *TG* mRNA expression was also decreased during proliferation but still detectable after 3–5 weeks; (5) the SAGM-grown cells were propagated from sorted TPO^{hi} cells.

Although both cytokeratin-18 and vimentin expressions were observed even in undifferentiated thyroid cancer cell lines, the cytokeratin-18 expression was lost in the SAGM-grown cells. All of thyroid-specific gene expressions were not observed in the cells. Moreover, the SAGM-grown cells displayed high plasticity: multilineage differentiation potential into thyrocytes, neuronal cells and adipocytes. These results suggest that we have successfully dedifferentiated/converted thyroid follicular cells into multilineage progenitor cells. However, re-differentiation effects (expression level of thyroid-specific genes) were modest compared to PT. We are currently seeking the better method enabling more efficient differentiation. In addition, these cells do not have unlimited proliferative capacity: the cells will stop growing until 3–4 months, due to cellular senescence.

Conversion of differentiated cells into multipotent progenitor or different lineage has been reported by several groups. Adult hepatocytes were converted into insulin-producing cells by transgenes, *Pdx-1* and *Ngn-3* [26]; retinal pigment epithelium into retinal neurons by *Sox-2* [27]; and interfollicular epidermal basal keratinocytes into the cells capable of differentiating into neuronal cells by *Oct-4* [28]. The aforementioned studies used exogenous trangenes, some of which are key transcription factors for generating iPS cells. To our knowledge, there is only one report describing conversion from differentiated cells into multilineage progenitor cells without any gene delivery. Adult intestinal epithelial cells were dedifferentiated into nestin-positive cells that have multilineage differentiation capacity into neuronal, pancreatic and hepatic lineages [29]. The authors cultured intestinal epithelial cells on mouse embryonic fibroblasts (feeder layer) in medium supplemented with leukemia inhibitory factor, EGF and bFGF. For generating iPS cells, two key transcription factors Klf-4 and Sox-2 could be replaced by chemical compounds [30,31].

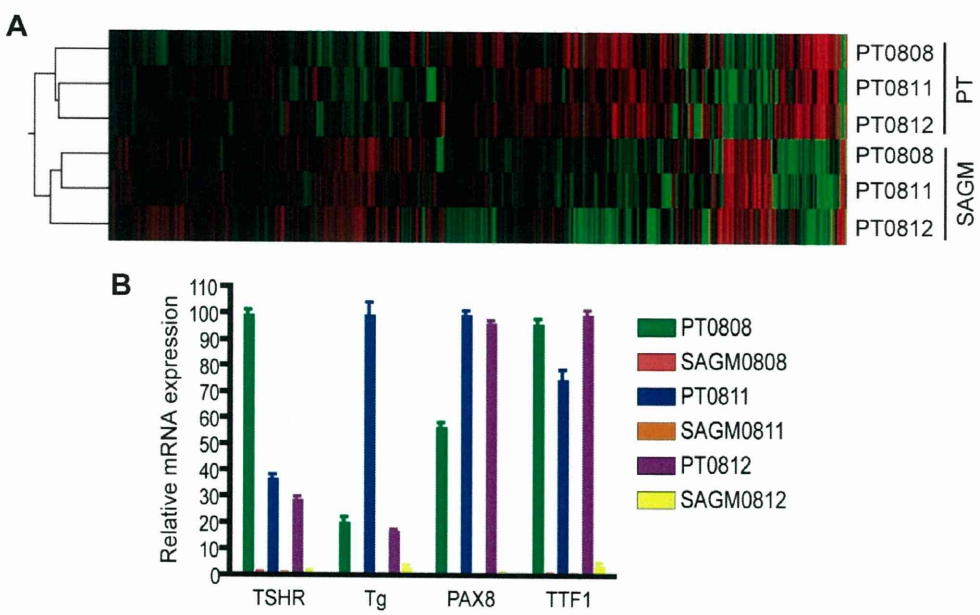

Figure 4. Microarray analysis of PT and corresponding SAGM-grown cells. A, The tree view of hierarchical clustering. PT: total RNA was extracted from PT on the next day of plating. SAGM: the SAGM-grown cells were expanded for two weeks and subcultured. On the next day, total RNA was extracted. The total RNAs were subjected to Affymetrix GeneChip Human Genome U133 Plus 2.0 microarray analysis service. Hierarchical clustering (by Manhattan distance) was performed using probes with "present call" only. Red columns represent higher expression, and green indicate lower expression. B, Confirmation of microarray data. Total RNA was subjected to qRT-PCR for indicated genes. Each bar indicates the mean and SD of the data collected in triplicate.
doi:10.1371/journal.pone.0019354.g004

Table 3. Fold-change of interested genes (SAGM cells/PT).

		PT0808			PT0811			PT0812		
Gene	**Probe**	**PT-flag**	**SAGM-flag**	**fold**	**PT-flag**	**SAGM-flag**	**fold**	**PT-flag**	**SAGM-flag**	**fold**
ABCG2	209735_at	A	A		A	A		A	A	
Oct4	208286_x_at	A	A		A	A		A	A	
CD133	204304_s_at	P	A	0.00	P	A	0.03	P	A	0.04
TG	203673_at	P	A	0.05	P	A	0.00	P	A	0.19
TSHR	210055_at	P	A	0.04	P	A	0.16	P	A	0.04
	215442_s_at	P	P	0.06	P	A	0.25	P	P	0.20
	215443_at	P	P	0.06	P	A	0.02	P	A	0.05
	237349_at	A	A		A	A		A	A	
PAX8	121_at	P	P	0.06	P	P	0.05	P	P	0.11
	207921_x_at	P	A	0.19	P	A	0.09	P	P	0.24
	207923_x_at	P	A	0.06	P	A	0.01	P	A	0.14
	207924_x_at	P	A	0.14	P	A	0.01	P	A	0.10
	209552_at	P	P	0.04	P	A	0.01	P	P	0.03
	213917_at	P	A	0.13	P	A	0.02	P	A	0.03
	214528_s_at	P	A	0.02	P	A	0.02	P	A	0.07
	221990_at	P	A	0.12	P	A	0.00	P	A	0.06
TPO	210342_s_at	P	A	0.34	P	A	0.03	P	A	0.62
TTF1	210673_x_at	P	A	0.01	P	A	0.02	P	A	0.44
	211024_s_at	P	P	0.04	P	P	0.04	P	P	0.12
CD106	203868_s_at	A	P	4.41	P	A	1.85	A	P	6.61
CD105	201808_s_at	A	A		A	A		A	A	
	201809_s_at	A	P	6.67	A	P	5.31	P	P	2.57
	228586_at	A	A		A	A		A	A	
CD90	208850_s_at	P	P	13.59	A	P	859.78	A	P	76.16
	208851_s_at	P	P	9.70	P	P	51.57	M	P	47.39
	213869_x_at	P	P	10.58	A	P	39.91	A	P	23.06
CD73	203939_at	P	P	0.83	P	P	1.27	P	P	1.50
	227486_at	P	P	1.22	P	P	1.46	P	P	1.18
	1553994_at	P	P	0.74	P	P	1.19	P	P	1.26
	1553995_a_at	P	P	0.70	P	P	1.16	P	P	1.24
KRT18	201596_x_at	P	P	0.02	P	P	0.02	P	P	0.31
Vim	201426_s_at	P	P	1.37	P	P	1.21	P	P	1.31
	1555938_x_at	P	P	0.75	P	P	1.00	P	P	0.46
E-CAD	201130_s_at	P	A	0.19	P	A	0.12	P	A	0.25
	201131_s_at	P	P	0.03	P	P	0.02	P	P	0.25

Flag score; P: present, M: marginal, A: absent.
doi:10.1371/journal.pone.0019354.t003

SAGM contains EGF, insulin, other growth factors and chemicals. These findings suggest that a particular combination of growth factors/chemicals probably changes transcriptional profiling, leading to dedifferentiation of thyrocytes.

Microarray analysis revealed that the expression of *ABCG2*, *Oct-4* and *CD133* which have been used to identify stem cells in a number of tissues, were not up-regulated in the SAGM-grown cells, suggesting that the cells were a bit far from genuine pluripotent stem cells. Rather, they seem to be more committed stem/progenitor cells. In fact, mesenchymal stem cell markers *CD106*, *CD105* and *CD90* but not *CD73* were highly up-regulated. Taken together with down-regulation of *E-cadherin*, these results suggest that the SAGM-grown cells acquired properties prior to cells committed to thyroid epithelial lineage.

GO enrichment analysis was performed and revealed differentially regulated cellular processes. Embryo implantation had the lowest p value among the up-regulated GO processes. Other up-regulated processes were endoplasmic reticulum, sterol/steroid/lipid biosynthesis/metabolic processes, cell cycle and oxidoreductase activity. These processes are generally related to protein/membrane synthesis presumably linked to cell proliferation. Regarding down-regulated GO processes, protein binding and cell adhesion processes were impacted. The down-regulation of cytoskeletal protein binding process might reflect to the loss of