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The acquisition of malignant potential in colon cancer is regulated by the stabilization of Atonal homolog 1 protein

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ABSTRACT

The transcription factor Atonal homolog 1 (Atoh1) plays crucial roles in the differentiation of intestinal epithelium cells. Although we have reported that the Atoh1 protein was degraded in colon cancer by aberrant Wnt signaling, a recent study has indicated that the Atoh1 protein is expressed in mucinous colon cancer (MC) and signet ring cell carcinoma (SRCC). However, the roles of the Atoh1 protein in MC are unknown. To mimic MC, a mutated Atoh1 protein was stably expressed in undifferentiated colon cancer cells. Microarray analysis revealed the acquisition of not only the differentiated cell form, but also malignant potential by Atoh1 protein stabilization. In particular, Atoh1 enhanced Wnt signaling, resulting in the induction of Lgr5 as a representative stem cell marker with the enrichment of cancer stem cells. Moreover, the fluorescent ubiquitination-based cell cycle indicator system with time-lapse live imaging demonstrated cell cycle arrest in the G0/G1 phase by Atoh1 protein stabilization. In conclusion, the Atoh1 protein regulates malignant potential rather than the differentiation phenotype of MC, suggesting the mechanism by which MC and SRCC are more malignant than non-mucinous adenocarcinoma.

1. Introduction

Pathological differentiation has been one of the most reliable histological criterion to predict the effectiveness of chemotherapy and the prognosis of colon cancer. Many reports support the idea that pathological differentiation is closely related to the progression and chemoresistance of a cancer [1]. However, "pathological differentiation" has often been confused with cell differentiation because the classification of pathological differentiation is based on the ductal formation of cancer cells. Interestingly, cancer cells that maintain the differentiated form such as mucinous carcinoma (MC) and signet ring cell carcinoma (SRCC) have often been classified as undifferentiated tumors on the basis of pathological findings. Nevertheless, the cell differentiation mechanism of colon cancer has not been investigated. One of the most important genes for cell formation is the basic helix–loop–helix (bHLH) transcription factor, Atonal homolog 1 (Atoh1), which is essential for differentiation toward secretory lineages in the small and large intestine [2]. Previous reports have suggested that the Atoh1 gene was suppressed by Wnt signaling in some colon cancers [3]. Moreover, we have demonstrated that the Atoh1 protein was actively degraded in colon cancer by the ubiquitin proteasomal system resulting in the disappearance of the Atoh1 protein in colon cancer despite Atoh1 gene expression [4]. Collectively, the deletion of adenomatous polyposis coli (APC) in colon carcinogenesis causes Atoh1 protein degradation by switching it to become the target of glycogen synthase kinase 3β GSK3β rather than β-catenin, resulting in maintenance of the undifferentiated cellular state [4]. On the other hand, it has been reported that the Atoh1 protein was expressed in MC and SRCC, both of which have secretory capacity [5]. It is notable that MC and SRCC are often classified as poorly differentiated tumors from pathological findings, resulting in a poorer

Abbreviations: Atoh1, Atonal homolog 1; MC, mucinous colon cancer; SRCC, signet ring cell carcinoma; GSK3, glycogen synthase kinase 3; Lgr5, Leucine-rich repeat-containing G-protein coupled receptor 5; IEC, intestinal epithelial cells; MUC2, mucin 2; TFF3, trefoil factor 3; HD6, human defensin 6; TCF4, T-cell factor 4; RT-PCR, reverse-transcription polymerase chain reaction; RLU, relative light units.

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prognosis than sporadic colon cancer. The relationship between pathological differentiation and cellular differentiation in colon cancer has not been clarified. Moreover, the significance of Atoh1 expression in cancer cells for malignant potential is controversial. It has been reported that the deletion of Atoh1 prevents cerebellar neoplasia in a mouse model of medulloblastoma in brain [6], suggesting that Atoh1 acts as a tumor accelerator. In contrast, Atoh1 is reported to be a tumor suppressor gene in Merkel cell carcinoma and colon cancer because Atoh1 suppressed cell proliferation [7]. However, there is no evidence except for the suppression of cell proliferation to confirm that in colon cancer Atoh1 acts as a tumor suppressor. Moreover, previous studies of Atoh1 function in colon cancer have analyzed the Atoh1 gene expression; however, because the expressed Atoh1 protein is degraded, its function has not been elucidated in detail.

In this study, we investigated the functions of Atoh1 in colon cancer to clarify the definition of cancer cell differentiation. We demonstrate that stable expression of the Atoh1 protein in colon cancer induces not only differentiation, but also the promotion of the malignant potential of colon cancer.

2. Materials and methods

2.1. Cell culture and chemicals

Sporadic human colon cancer-derived SW480, DLD1 cells and human embryonic kidney-derived 293T cells were cultured as described previously [8] Plasmid DNA was transfected as described previously [8]. Lentivirus infection was performed according to the manufacturer's protocols. The infected cell lines were supplemented with Blasticidin (7.5 μg/ml, Invitrogen Carlsbad, CA, USA) during maintenance. Oxaliplatin (L-OHP) was used (Tocris Cookson, Ellisville, MI, USA) for evaluating chemoresistance.

2.2. Plasmids

The mCherry-Atoh1 vector was generated by inserting Atoh1 gene into the mCherry DNA template PG27188 (DNA 2.0, Menlo Park, CA, USA). The Atoh1 mutant (5SA-Atoh1) was constructed by PCR-mediated mutagenesis in which five serine residues, TCC (160–162) and AGC (172–174, 328–330, 340–342, 352–354), were replaced with alanine residue GCC. The Atoh1-lentivirus vector was generated by inserting the PCR-amplified mCherry-Atoh1 gene or mCherry-5SA-Atoh1 into pLenti 6.4 (Invitrogen). The S/G2/M-green-lentivirus vector was generated by inserting the PCR-amplified pFucci-S/G2/M-green DNA sequence into pLenti 6.4 (Invitrogen). The lentivirus was generated according to the procedure manual. 5′ Lgr5 reporter plasmid was generated by cloning a 1000 bp sequence 5′of the human Lgr5 gene into a pGL4 basic vector (Promega, Madison, WI). The promoter region of Lgr5 was gradually shortened by 200–1000 bp was generated into a pGL4 basic vector. Polymerase chain reaction-mediated mutagenesis was used to construct internal deletion mutants of the 5′ Lgr5 reporter plasmid in which the following base pair sets −300 to −310, −310 to −320, −320 to −330, −330 to −340, and −340 to −350 were deleted separately.

2.3. Quantitative real-time PCR

Total RNA was isolated with TRIzol® reagent (Invitrogen) according to the manufacturer's instructions as described previously [8]. The primer sequences in this study are summarized in Supplementary Table S1. In all examinations, the expression in LS174T cells (mucinous phenotype colon cancer cell line) was used as standard.

2.4. Western blot analysis

Cells were extracted with 1% sodium dodecyl sulfate (SDS)-containing radioimmunoprecipitation assay (RIPA) buffer as described previously [8]. The membranes were immunoblotted with anti-mCherry (Clontech, Mountain View, CA, USA) and anti-USF2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies before incubation with secondary antibodies.

2.5. Immunofluorescence analysis

The cells were fixed as described previously [8]. The antibodies used were anti-human MUC2 (Ccp58; Santa Cruz Biotechnology), anti-human TFF3 (ab57752; Abcam, Cambridge, UK) and anti-human Lgr5 (TA301323; OriGene, Rockville, MD, USA). Anti-mouse IgG Alexa Fluor® 594 or Alexa Fluor® 488 (Invitrogen) were used as the secondary antibody. Cells were mounted with VectaShield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and visualized by confocal laser fluorescent microscopy (BZ-8000 [Keyence, Tokyo] and FLUOVIEW FV10i [Olympus, Tokyo]).

2.6. MTS assay

1×10^4 DLD1 cells were cultured in a 96-well tissue culture plate at 37 °C, and 5% CO_2. After a 48-h incubation period in the presence or absence of oxaliplatin, CellTiter96® Aqueous One Solution was added (20 μl/well) and incubated for 1 h at 37 °C and 5% CO_2. The absorbances at 490 nm were measured with an ARVO™MX plate reader (Perkin Elmer, Boston, MA, USA). Background absorbances from medium-containing wells were subtracted from those of the sample wells.

2.7. Migration assay

The Oris™ Pro Cell Migration Assay kit (Platypus Technologies, LLC USA) was used. This assay is formatted for 96 well-plates and uses a non-toxic biocompatible gel to form a cell-free zone on cell culture surfaces. DLD1 cells (1×10^4) were seeded into 96-well plates and incubated for 1 h. Phase contrast images were taken for pre-migration reference. After 6 h of incubation, images were captured using phase contrast microscopy. The ratio of the vacant area between pre- and post-migration was analyzed.

2.8. Cell cycle assay and live cell imaging

Live imaging was performed on the DeltaVision system (Applied Precision, Washington, USA) incorporating a fluorescent microscope IX-71 (Olympus, Tokyo, Japan) using a 20× 0.75NA Olympus UPlanSApo objective. Differential interference contrast (DIC) and fluorescent images were acquired at 15-min intervals for 72 h. The data were processed using softWoRx® (Applied Precision). Maximum intensity projections of the time series were exported into QuickTime format for presentation as Supplementary movies. The ratio of cells in the S/G2/M phase was analyzed by FACS caliber to detect cells expressing S/G2/M green fluorescence.

2.9. Chromatin immunoprecipitation assay

A chromatin immunoprecipitation (ChIP) assay was performed as described previously with some modifications [9]. The primer sequences in this study are summarized in Supplementary Table S1.

2.10. Statistical analysis

Quantitative real-time PCR analyses were statistically analyzed with the Student's *t*-test. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. Mutant Atoh1 protein stabilized in colon cancer acquires mucinous secretion and Lgr5 expression

To assess the function of the Atoh1 protein in colon cancer, we attempted to construct a mutant Atoh1 protein that was stably expressed in undifferentiated colon cancer cells. In our previous study, the SA-Atoh1 protein, in which two serine residues were replaced by alanine, was transiently expressed in colon cancer cells, leading to a slight increase in mucin 2 (MUC2) gene expression [8]. However, SA-Atoh1 was not stably expressed in colon cancer cells (data not shown). Therefore, a mutant Atoh1 gene (5SA-Atoh1) in which five serine residues were replaced by alanine was generated in order to avoid phosphorylation by GSK3 and proteasomal degradation in colon cancer cells (Supplementary Fig. S1). Both of these Atoh1 proteins were expressed in 293T cells, in which Wnt signaling is normal. In contrast, only the 5SA-Atoh1 protein was stably expressed in sporadic colon cancer derived DLD1 cells and SW480 cells (Fig. 1A). Fluorescence analysis showed the expression of 5SA-Atoh1 in the nuclei of DLD1 cells (Fig. 1B). Moreover, the transcriptional activity of 5SA-Atoh1 was conserved through the E-box sequence even in DLD1 cells (Fig. 1C). Atoh1 protein stabilization also resulted in the induction of the secretory phenotypic genes and proteins such as MUC2, trefoil factor 3 (TFF3) and human defensin 6 (HD6) (Fig. 1D and E). The expression in LS174T cells that are mucinous colon cancer cell line were used as standard in all PCR examination, since the quantity of gene expression in LS174T cells were defined as 1. Thus, Atoh1 protein-expressing cells were generated for the first time in colon cancer, resulting in the acquisition of a mucinous phenotype, although MUC2 expression in Atoh1 expressing cells was lower than in LS174T.

To assess whether the expression of the Atoh1 protein affects the malignant potential of colon cancer, comprehensive genes induced by the Atoh1 protein were detected by microarray analysis. A gene set enrichment analysis (GSEA) indicated the malignant potential by identifying genes involved in the Wnt pathway, cell adhesion, cell cycle arrest, and metastasis in 5SA-Atoh1 cells (Supplementary Table S2). Moreover, cancer stem markers upregulated by Atoh1 were found in the microarray gene list (Supplementary Table S3). Therefore, we confirmed the expression of cancer stem markers in 5SA-Atoh1 cells. Interestingly, a leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) gene was markedly increased in 5SA-Atoh1 DLD1 cells (Fig. 1F). These findings that stabilized Atoh1 protein induces both mucinous phenotype and Lgr5 gene were also shown in SW480 cells (data not shown).

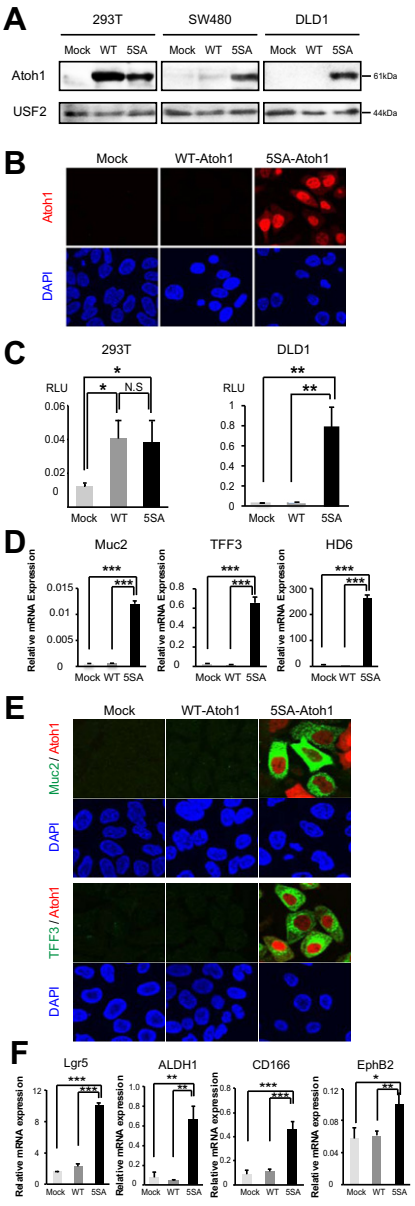

Fig. 1. Mutant Atoh1 protein stabilized in colon cancer acquires mucinous secretion and Lgr5 expression. (A) WT-Atoh1 and 5SA-Atoh1 were transfected into 293T, SW480, and DLD1 cells. The protein expression of WT-Atoh1 and 5SA-Atoh1 was analyzed by Western blot. (B) Immunofluorescence staining showed the nuclear localization of the 5SA-Atoh1 protein in DLD1 cells. (C) A reporter activity via the E-box sequence (E-box-Luc) was analyzed. In DLD1 cells, 5SA-Atoh1 induced significantly higher reporter activity than WT-Atoh1, with correspondingly higher protein expression. (D) The expression of the differentiation phenotypic genes was analyzed by RT-PCR. The MUC2 TFF3 and HD6 genes were significantly upregulated in 5SA-Atoh1 DLD1 cells. The expression level in LS174T cells was defined as 1, (E) Immunofluorescence staining of MUC2 and TFF3 showed the expression of both proteins only in 5SA-Atoh1 DLD1 cells and (F) The expression of the cancer stem cell marker genes was analyzed by RT-PCR. Lgr5 was significantly upregulated in 5SA-Atoh1DLD1 cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 3$.

3.2. Atoh1 protein directly upregulates the transcriptional activity of Lgr5

The Lgr5 protein was detected in peripheral nuclei and the cell membrane showed a punctate appearance, consistent with a previous report (Fig. 2A) [10]. As Lgr5 is one of the Wnt target genes, we assessed Wnt signaling activity by examining T cell factor 4 (TCF4)-dependent transcriptional activity in 5SA-Atoh1 cells. TCF4 transcriptional activity was upregulated in 5SA-Atoh1/DLD1 cells but not in WT-Atoh1 cells (Fig. 2B), suggesting that Atoh1 has the potential to promote transcription through the TCF4 binding site. The promoter assay with human Lgr5 also showed an upregulation by the Atoh1 protein. The next critical region on the Lgr5 promoter was limited to within 350 bp upstream (Fig. 2C). Finally, deletion mutants of the Lgr5 promoter revealed that the critical region for transcription by Atoh1 lay between 340 bp and 350 bp upstream (Fig. 2D). Because, The transcriptional activity for Lgr5 was also upregulated by Atoh1 expressed transiently in 293T cells

(Fig. 2E), We confirmed the binding of Atoh1 to the Lgr5 promoter region by ChIP assay (Fig. 2F and G), suggesting that Atoh1 directly upregulates Lgr5 promoter activity in the region located between 340 bp and 350 bp upstream of Lgr5.

3.3. Atoh1 protein stabilization enriches the cancer stem cells in vitro

We next assessed the cancer stemness by Atoh1 protein stabilization, because various stem cell markers including Lgr5 were induced

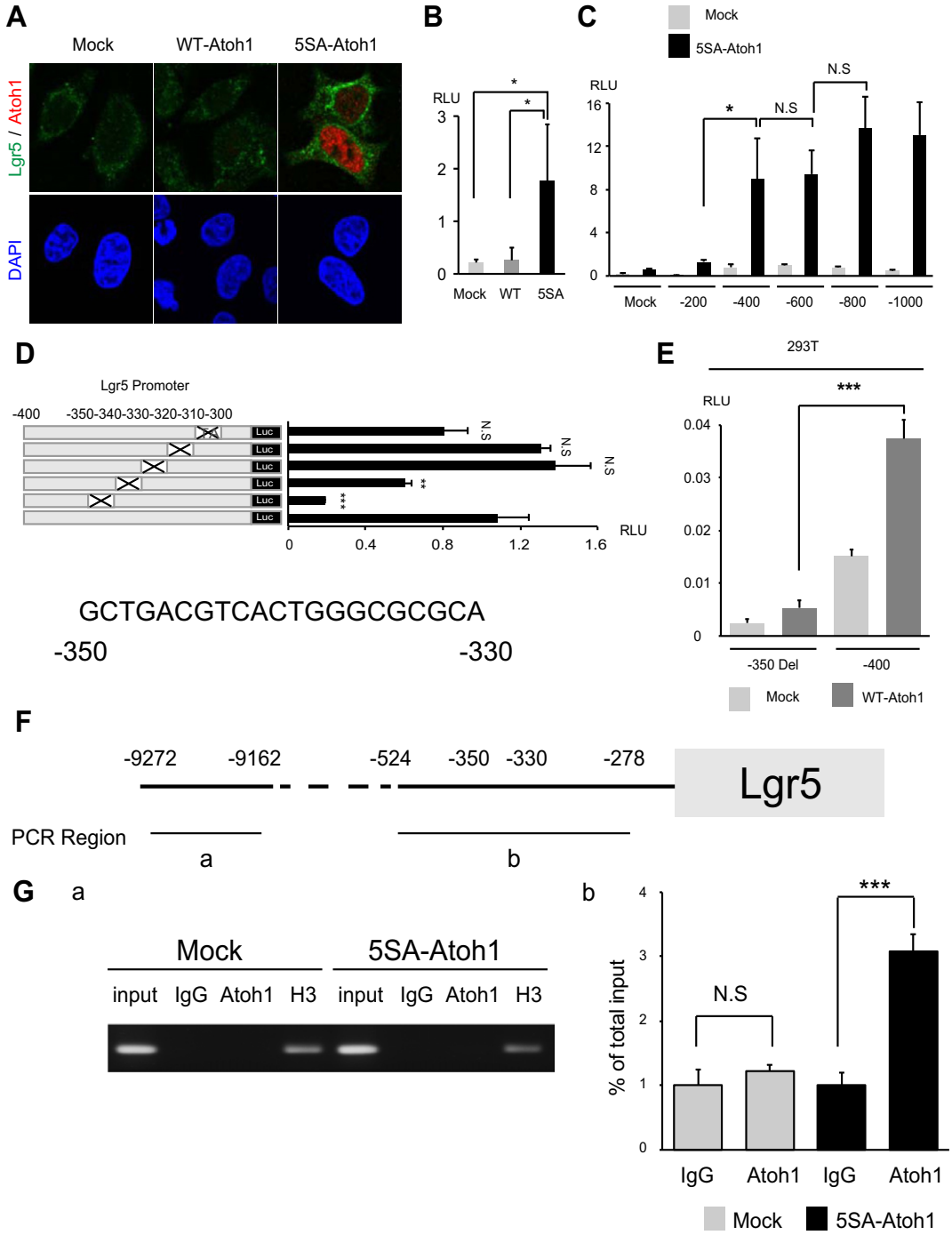

Fig. 2. Atoh1 protein directly upregulates the transcriptional activity of Lgr5. (A) Immunofluorescence staining showed the expression of Lgr5 in 5SA-Atoh1/DLD1 cells. (B) TCF4-dependent reporter activity (TOPflash) was examined. Reporter activity was significantly upregulated in 5SA-Atoh1 DLD1 cells. (C) The reporter activity of Lgr5 promoters that were longer than 400 bp was elevated only in 5SA-Atoh1 DLD1 cells. (D) Mutated Lgr5 reporter vectors each with a 10-bp deleted inform from 300 to 350 bp upstream of the Lgr5 promoter region were generated. The reporter activity by Atoh1 protein expression was significantly decreased between 340 bp and 350 bp of the Lgr5 promoter. The sequence between 330 bp and 350 bp of Lgr5 promoter was shown. (E) WT-Atoh1 upregulated the transcriptional activity through the region between 340 bp and 350 bp of the promoter region in 293T cells. (F) Schematic representation of the human Lgr5 genome and the region amplified by PCR and (G) the ChIP assay was performed using DLD1 cells with or without 5SA-Atoh1. Each region indicated by a schema was amplified from the immunoprecipitant by each antibody. Only the region including the 340–350-bp segment of the Lgr5 promoter (region b) was amplified from the immunoprecipitant by the mCherry antibody. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 3$.

Table 1
Frequency of spheroid-forming cells in DLD1 or 5SA-Atoh1/DLD1 cells by extreme limiting dilution analysis. Various concentrations of DLD1 cells expressing GFP or 5SA-Atoh1 (1000, 500, 100, and 10) were cultured with SCM medium in low attachment dishes. Ten days after, the number of spheroids was counted. The confidence interval (95%) for spheroid forming frequency was calculated using a software application for limiting dilution analysis (LDA), ELDA, as described previously [11].

No. of cells	No. of spheroids	
	Naive	5SA-Atoh1
1000	28.3	50.3
500	10.6	31
100	5.6	13.6
10	0.6	5
Spheroid-forming frequency (95% confidence interval)	1/27559 (1/36913–1/20576)	1/12273 (1/14946–1/10079)
P value	2.75×10^{-6}	

by Atoh1. *In vitro* spheroid formation assay showed the tumorigenicity in 5SA-Atoh1 cells derived spheroids. Analysis by extreme limiting dilution analysis (ELDA) [11] showed that the Atoh1 protein enriched cancer stem cells (Table 1) (Supplementary Fig. S2).

3.4. Atoh1 protein stabilization suppresses cell growth by extension of the G0/G1 phase of the cell cycle

To further analyze the functional role of the Atoh1 protein in colon cancer cells, we examined whether the Atoh1 protein affected cell proliferation. Atoh1 protein stabilization suppressed cell proliferation in all colon cancer cells (Fig. 3A). A previous report has suggested that the Atoh1 protein in Merkel cell carcinoma induces apoptosis, resulting in the suppression of cell proliferation [7]. However, Atoh1 protein stabilization did not induce an apoptotic signal in DLD1 cells (data not shown). As cell cycle may affect cell proliferation in cells expressing the Atoh1 protein, we attempted to visualize the cell cycle using a fluorescent ubiquitination-based cell cycle indicator (Fucci) system in which cells in the S/G2/M phase were marked by Azami-Green 1 fused with geminin [12]. Live-imaging analysis showed an extension of the G0/G1 phase in 5SA-Atoh1 cells, resulting in a longer cell cycle by Atoh1 protein stabilization (Fig. 3B; Supplementary Table S4; Supplementary Movie S1, 2). Moreover, a decreased number of 5SA-Atoh1 cells in the S/G2/M phase were found compared with mock-transfected cells (Fig. 3C).

3.5. Atoh1 protein stabilization promotes cell migration and chemoresistance in colon cancer cells

5SA-Atoh1 cells were transformed cell shape and cell adhesion that were dispersed each cells (Supplementary Movie S2). Cells at rest exhibited accelerated migration (Fig. 3D and E), indicating that the Atoh1 protein may confer not only the secretory phenotype but also a cancer stem cell phenotype. We finally assessed whether the Atoh1 protein conferred chemoresistance. 5SA-Atoh1 DLD1 cells were more resistant to oxaliplatin than both WT-Atoh1 DLD1 cells and mock-transfected DLD1 cells. WT-Atoh1 protein was also expressed by the treatment with oxialiplatin because of the GSK3 inactivation (Supplementary Fig. 3), resulting in the resistance to oxaliplatin in WT-Atoh1 DLD1 cells (Fig. 3F).

4. Discussion

This study revealed that the mutated Atoh1 protein could be stably expressed in colon cancer cells. We also demonstrated that Atoh1 protein stabilization induces both a cancer stem cell phenotype and a mucinous phenotype, resulting in the acquisition of chemoresistance.

One of the most reliable markers for cancer stem cells is Lgr5 because Lgr5 is essential for the maintenance of intestinal epithelial stem cells [13]. Lgr5-positive colon cancer cells have the potential to become cancer stem cells because the Lgr5 gene is selectively expressed in human colon cancer stem cell populations [14,15]. Recently, Lgr5-positive cells were reported to be stem cells in adenoma by a lineage tracing method [16], supporting that Lgr5 may be one of the cancer stem markers in the colon. Although it remains to be elucidated how the Atoh1 protein induces the Lgr5 gene expression, this study showed that the Atoh1 protein directly induces the promoter activity of the Lgr5 gene, resulting in the enrichment of cancer stem cells. Whether Lgr5 induced by Atoh1 is related to the enrichment of cancer stem cells should be clarified more in detail in future.

Another interesting finding was that the Atoh1 protein extended the G0/G1 phase of the cell cycle. Atoh1 gene expression is known to be negatively regulated by the Notch signal [9], which promotes the cell cycle [17]. Therefore, it has been considered that cell cycle arrest during intestinal epithelial cell differentiation could be attributed to Notch signal suppression rather than Atoh1 gene expression. However, a recent study showed that deletion of the Atoh1 gene canceled the suppression of cell proliferation by Notch signal inhibition in colon cancer [18], indicating that Atoh1 may directly regulate cell proliferation. It was only after stabilization of the Atoh1 protein in colon cancer that cell proliferation was markedly suppressed *in vitro*. Because some reports have indicated that slow cycling cancer stem cell populations was included in tumor to survive therapies, cell cycle arrest by Atoh1 might express one of phenotypes for cancer stem cells [19].

Moreover, the Atoh1 protein induced chemoresistance by the avoidance of G2 phase entry, which most alkylating agents target [20]. These results provide useful information on the acquisition of chemoresistance by MC and SRCC that express the Atoh1 protein. Furthermore, WT-Atoh1 DLD1 cells were also resistance to oxaliplatin by the stabilization of Atoh1 protein. Therefore, mRNA expression of Atoh1 in non-mucinous sporadic colon cancer might be important to assess the chemoresistance and how oxaliplatin stabilizes Atoh1 protein should be clarified in future.

The reason why cell cycle arrest does not induce apoptosis has an important bearing on the significance of Atoh1 protein expression in cancer cells. A major difference in Atoh1 protein expression between normal tissue and cancer cells may be the Wnt signaling. β-Catenin and Atoh1 do co-localize in the nuclei of mucinous carcinoma, probably because of GSK3 inactivation, resulting in the increase of TCF4-dependent transcriptional activity in cooperation with β-catenin. Consequently, Lgr5 and MMP9 (data not shown), both of which contribute to cell survival [21], were upregulated by Wnt signaling acceleration with Atoh1 protein stabilization, suggesting that cancer cells may prevent apoptosis in spite of the cell cycle arrest. Although it is unknown how the Atoh1 protein is permanently expressed in mucinous cancer, the investigation of GSK3 kinase inactivation by GSK3 phosphorylation in mucinous cancer may help in clarifying the mechanism by which the Atoh1 protein is stabilized.

Overall, Atoh1 protein stabilization appears to act as a tumor suppressor that differentiates colon cancer cells by inducing differentiation phenotypes and suppressing cell proliferation. Nevertheless, the Atoh1 protein conferred cancer stem cell phenotypes, such as cell migration, cancer stem marker expression, and chemoresistance, indicating that in colon cancer, the Atoh1 protein plays a greater role in malignant potential than tumor suppression. The

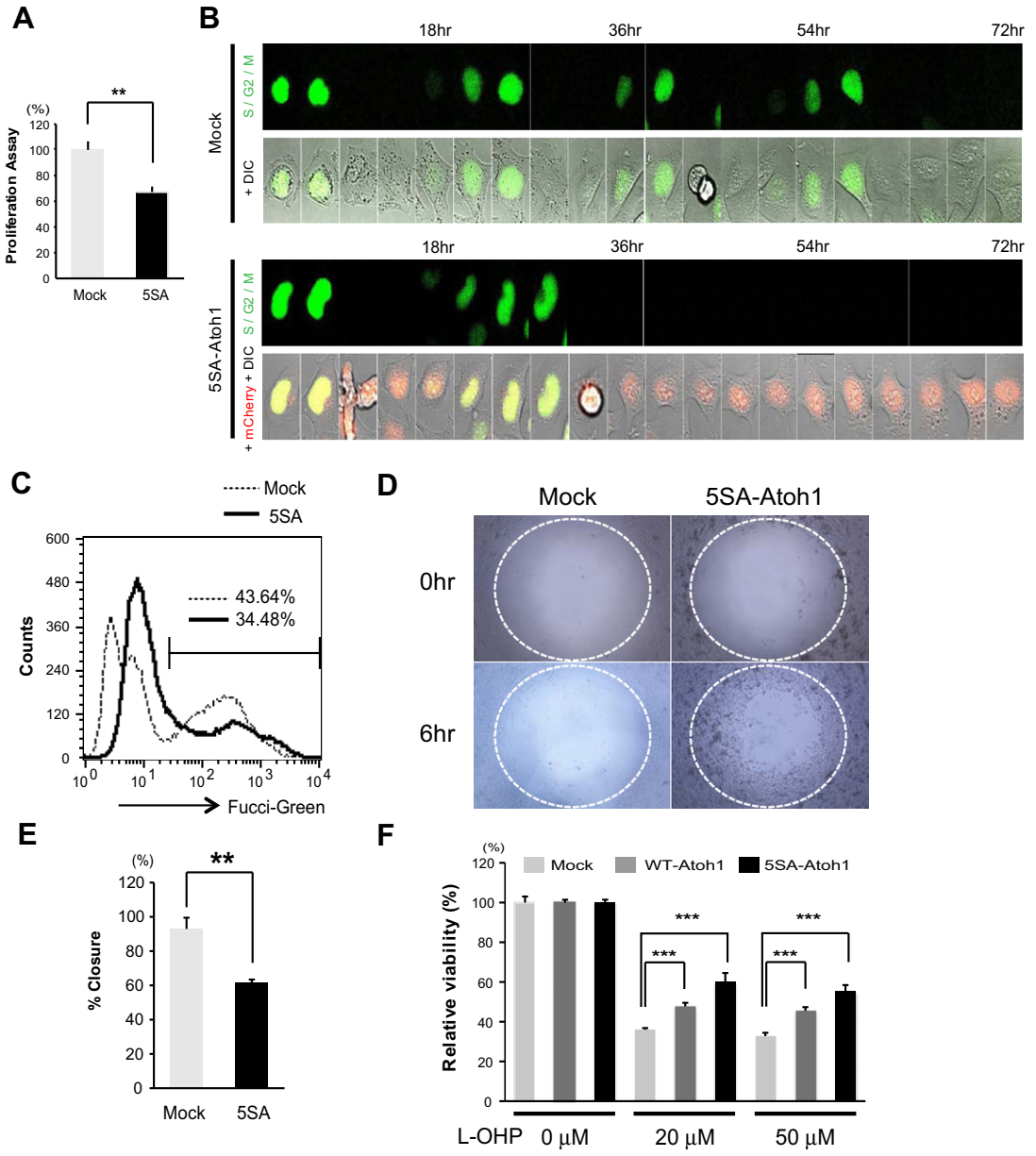

Fig. 3. Atoh1 protein stabilization in colon cancer induces cell cycle arrest, migration and chemoresistance. (A) A proliferation assay revealed that cells expressing 5SA-Atoh1 grew slowly. (B) To visualize the S/G2/M phase of cells, a lentivirus composed of Azami-Green combined with geminin (Green-S/G2/M) was infected into both DLD1 cells and 5SA-Atoh1 DLD1 cells. Time-lapse live imaging of DLD1 cells showed elongation of the G0/G1 phase in 5SA-Atoh1 DLD1 cells. (C) FACS analysis showed a decreased number of 5SA-Atoh1/DLD1 cells in the S/G2/M phase, resulting in the accumulation of 5SA-Atoh1 DLD1 cells in the G0/G1 phase. (D) Migration assay showed that the vacant circular area was occupied by a greater number of 5SA-Atoh1 DLD1 cells than DLD1 cells. (E) The ratio of the remaining vacant area is shown. The vacant area of 5SA-Atoh1 DLD1 cells was smaller than that of DLD1 cells at 6 h after the cells were seeded and (F) An MTS assay showed that the reduction of cells in both 5SA-Atoh1 DLD1 cells and WT-Atoh1 DLD1 cells by treatment with oxaliplatin (L-OHP) was less than that of DLD1 cells. (**$p < 0.01$, ***$p < 0.001$, N.S.: not significant). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

present study also suggests that the cell differentiation phenotype regulated by Atoh1 expression in colon cancer is not linked to the pathological differentiation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.034.

References

[1] J.R. Jass, B.C. Morson, Reporting colorectal cancer, J. Clin. Pathol. 40 (1987) 1016–1023.

[2] Q. Yang, N.A. Bermingham, M.J. Finegold, H.Y. Zoghbi, Requirement of Math1 for secretory cell lineage commitment in the mouse intestine, Science 294 (2001) 2155–2158.

[3] C.C. Leow, M.S. Romero, S. Ross, P. Polakis, W.Q. Gao, Hath1, down-regulated in colon adenocarcinomas, inhibits proliferation and tumorigenesis of colon cancer cells, Cancer Res. 64 (2004) 6050–6057.
[4] K. Tsuchiya, T. Nakamura, R. Okamoto, T. Kanai, M. Watanabe, Reciprocal targeting of Hath1 and beta-catenin by Wnt glycogen synthase kinase 3 beta in human colon cancer, Gastroenterology 132 (2007) 208–220.
[5] E.T. Park, H.K. Oh, J.R. Gum, S.C. Crawley, S. Kakar, J. Engel, C.C. Leow, W.Q. Gao, Y.S. Kim, HATH1 expression in mucinous cancers of the colorectum and related lesions, Clin. Cancer Res. 12 (2006) 5403–5410.
[6] O. Ayrault, H. Zhao, F. Zindy, C. Qu, C.J. Sherr, M.F. Roussel, Atoh1 inhibits neuronal differentiation and collaborates with Gli1 to generate medulloblastoma-initiating cells, Cancer Res. 70 (2010) 5618–5627.
[7] W. Bossuyt, A. Kazanjian, N. De Geest, S. Van Kelst, G. De Hertogh, K. Geboes, G.P. Boivin, J. Luciani, F. Fuks, M. Chuah, T. VandenDriessche, P. Marynen, J. Cools, N.F. Shroyer, B.A. Hassan, Atonal homolog 1 is a tumor suppressor gene, PLoS Biol. 7 (2009) e39.
[8] M. Aragaki, K. Tsuchiya, R. Okamoto, S. Yoshioka, T. Nakamura, N. Sakamoto, T. Kanai, M. Watanabe, Proteasomal degradation of Atoh1 by aberrant Wnt signaling maintains the undifferentiated state of colon cancer, Biochem. Biophys. Res. Commun. 368 (2008) 923–929.
[9] X. Zheng, K. Tsuchiya, R. Okamoto, M. Iwasaki, Y. Kano, N. Sakamoto, T. Nakamura, M. Watanabe, Suppression of hath1 gene expression directly regulated by hes1 via notch signaling is associated with goblet cell depletion in ulcerative colitis, Inflamm. Bowel Dis. 17 (2011) 2251–2260.
[10] S. Kobayashi, H. Yamada-Okabe, M. Suzuki, O. Natori, A. Kato, K. Matsubara, Y. Jau Chen, M. Yamazaki, S. Funahashi, K. Yoshida, E. Hashimoto, Y. Watanabe, H. Mutoh, M. Ashihara, C. Kato, T. Watanabe, T. Yoshikubo, N. Tamaoki, T. Ochiya, M. Kuroda, A.J. Levine, T. Yamazaki, LGR5-positive colon cancer stem cells interconvert with drug-resistant LGR5-negative cells and are capable of tumor reconstitution, Stem Cells 30 (2012) 2631–2644.
[11] Y. Hu, G.K. Smyth, ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays, J. Immunol. Methods 347 (2009) 70–78.
[12] A. Sakaue-Sawano, H. Kurokawa, T. Morimura, A. Hanyu, H. Hama, H. Osawa, S. Kashiwagi, K. Fukami, T. Miyata, H. Miyoshi, T. Imamura, M. Ogawa, H. Masai, A. Miyawaki, Visualizing spatiotemporal dynamics of multicellular cell-cycle progression, Cell 132 (2008) 487–498.
[13] N. Barker, J.H. van Es, J. Kuipers, P. Kujala, M. van den Born, M. Cozijnsen, A. Haegebarth, J. Korving, H. Begthel, P.J. Peters, H. Clevers, Identification of stem cells in small intestine and colon by marker gene Lgr5, Nature 449 (2007) U1001–U1003.
[14] K. Kemper, P.R. Prasetyanti, W. de Lau, H. Rodermond, H. Clevers, J.P. Medema, Monoclonal antibodies against lgr5 identify human colorectal cancer stem cells, Stem Cells 30 (2012) 2378–2386.
[15] M. Leushacke, N. Barker, Lgr5 and Lgr6 as markers to study adult stem cell roles in self-renewal and cancer, Oncogene 31 (2011) 3009–3022.
[16] A.G. Schepers, H.J. Snippert, D.E. Stange, M. van den Born, J.H. van Es, M. van de Wetering, H. Clevers, Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas, Science 337 (2012) 730–735.
[17] E. Sancho, E. Batlle, H. Clevers, Signaling pathways in intestinal development and cancer, Annu. Rev. Cell Dev. Biol. 20 (2004) 695–723.
[18] A. Kazanjian, N.F. Shroyer, NOTCH signaling and ATOH1 in colorectal cancers, Curr. Colorectal Cancer Rep. 7 (2011) 121–127.
[19] N. Moore, S. Lyle, Quiescent, slow-cycling stem cell populations in cancer: a review of the evidence and discussion of significance, J. Oncol. 2011 (2011), http://dx.doi.org/10.1155/2011/396076.
[20] P. Bozko, M. Sabisz, A.K. Larsen, A. Skladanowski, Cross-talk between DNA damage and cell survival checkpoints during G2 and mitosis: pharmacologic implications, Mol. Cancer Ther. 4 (2005) 2016–2025.
[21] K. Kessenbrock, V. Plaks, Z. Werb, Matrix metalloproteinases: regulators of the tumor microenvironment, Cell 141 (2010) 52–67.