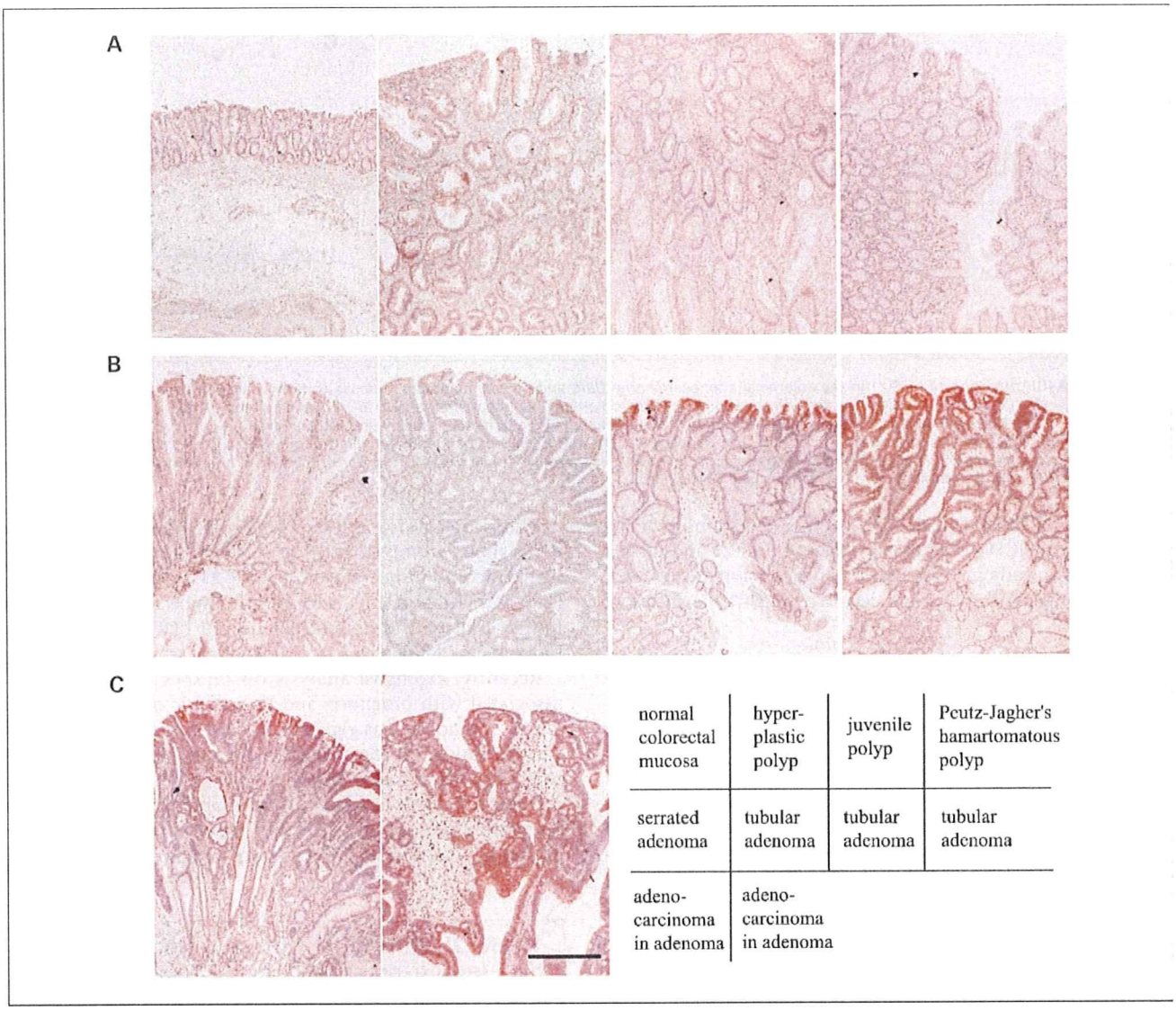

Fig. 3. LNA-ISH detecting miR-21 with FFPE colorectal tissues obtained from endoscopic mucosal resection. Bar, 500 μm. *A*, nontumorous colorectal tissues. Photos from left to right are normal colorectal mucosa, hyperplastic polyp, juvenile polyp, and Peutz-Jagher's hamartomatous polyp. *B*, tumorous but nonmalignant colorectal lesions. Photos from left to right are serrated adenoma (negative for miR-21), tubular adenoma (negative for miR-21), tubular adenoma (miR-21 positive in the edge), and tubular adenoma (positive for miR-21). *C*, tumorous and malignant colorectal lesions. In each photo, adenocarcinoma in adenoma, strongly positive for miR-21, is shown.

II or stage III colon cancer patients, indicating its potential as a prognostic biomarker (15).

LNA-ISH combined with biotin-free tyramide signal amplification system is a useful technique for determination of miRNA expression levels in FFPE tissues. The LNA-modified oligonucleotide would be one of the most sensitive probes currently available for miRNA detection (34, 35). Nevertheless, it is quite difficult to detect miRNAs by ISH, especially in FFPE clinical tissues. It has been reported that the LNA-ISH technique could detect some miRNA species in FFPE samples (36). However, using LNA-modified probes alone or LNA-ISH combined with a universal immunoenzyme polymer method (Histofine Simple Stain MAX PO_MULTI purchased from Nichirei), we were not able to detect miR-21 expression in FFPE samples (data not shown). In our previous study (31), the biotin-free tyramide signal amplification system was used to detect the nuclear protein Brm, which is difficult to detect by immunohistologic methods (37). In the present study, we applied this method to LNA-ISH and were able to sensitively detect miR-21 expression. Because detection of nonspecific signals is not infrequent when using tyramide amplification, we confirmed that staining by the scramble control probe (Supplementary Fig. S5B) as well as staining unrelated to the LNA/DNA probe (Supplementary Fig. S4B) was only rarely detected.

High-level expression of miR-21 in cancer-associated fibroblasts may be induced by secreting factors originating from cancer cells. It is very interesting that the stromal fibroblasts around tumors frequently express miR-21 at high levels. In most cases from the present study, these cancer-associated fibroblasts showed strong miR-21 expression compared with distant normal fibroblasts

and normal epithelial cells (Supplementary Table S1). In some cases, the fibroblast staining intensities were even more intense than those of adjacent malignant cells (Supplementary Fig. S4A). Therefore, we hypothesized that this is a non–cell-autonomous phenomenon and that cytokines secreted from the adjacent malignant tumors might contribute to miR-21 induction. In this regard, it has been reported that interleukin-6 levels are elevated in the cancer-associated fibroblasts around a colon cancer, in serum, and in tumor tissues from colorectal cancer patients (38, 39). It is also noteworthy that interleukin-6 induces the transcription of miR-21 in multiple myeloma cells through mediation of signal transducer and activator of transcription-3 activity (12). Importantly, signal transducer and activator of transcription-3 binding sites are present just upstream of the transcriptional start site in the *miR-21* promoter. Therefore, interleukin-6 is a candidate for miR-21 induction in these cancer-associated fibroblasts.

MiR-21 RNA and PDCD4 protein expression patterns were mutually exclusive in colorectal epithelial cells. PDCD4 was highly expressed in normal colorectal epithelium, but PDCD4 expression was often reduced in precancerous colorectal regions (Fig. 4). This observation is consistent with a recent report that normal mucosa showed strong nuclear PDCD4, which was significantly reduced in adenomas (40). Our ISH analyses further indicated that cells with reduced PDCD4 expression frequently had elevated miR-21 expression, which was nondetectable in normal colorectal epithelium (Fig. 4). In progressive colorectal cancers, almost all cells expressed miR-21, whereas PDCD4 was almost undetectable. In summary of our ISH analysis, expression of miR-21 RNA and PDCD4 protein showed mutually exclusive patterns in colorectal epithelial cells. These observations support that PDCD4 is a good target of miR-21 *in vivo*. Because PDCD4 is a potent tumor suppresser, miR-21 may perform oncogenic functions, at least in part, through down-regulation of PDCD4.

We have previously shown that in human cell culture systems, a double-negative feedback loop operates between miR-21 and its target protein, NFIB, through the miR-21 promoter, which has the binding site of this negative transcriptional regulator (16). This means that the *miR-21* gene integrates a system that self-reinforces its own expression. Whereas in adult rats *NFIB* mRNA is highly expressed (41), we are currently not able to perform specific immunohistochemical staining due to the absence of a specific anti-NFIB antibody that is applicable to FFPE clinical samples and is non–cross-reactive with other NFI family members. Therefore, direct evidence of NFIB involvement *in vivo* remains to be established. Using rat cell culture system, Dr. Verde's group very recently indicated that PDCD4 suppressed miR-21 promoter activity, at least in part, by inhibiting AP-1 activity

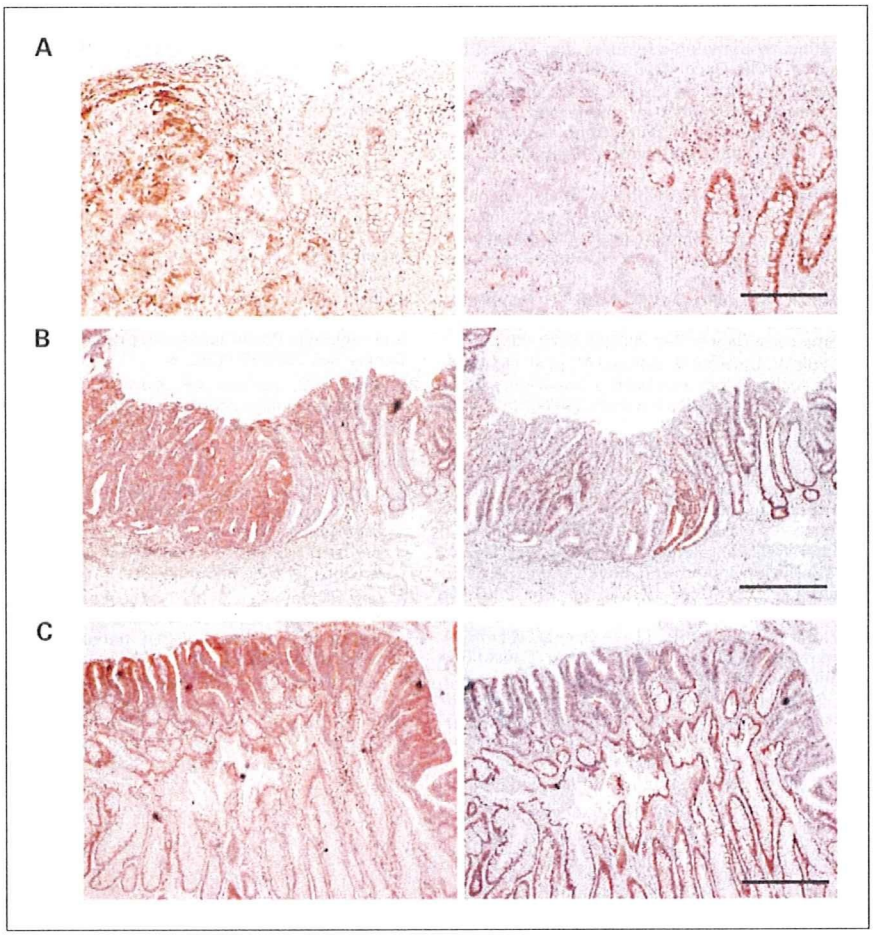

Fig. 4. Images from sequential FFPE colorectal tissue sections in which LNA-ISH for miR-21 RNA (*left*) and immunostaining for PDCD4 protein (*right*) were done. *A*, a surgically resected colorectal adenocarcinoma. The border of the colorectal cancer (*left half*) and normal colorectal mucosa (*right half*) is apparent in the two photos. Bar, 200 μm. *B*, a colorectal polyp containing adenocarcinoma and adenoma obtained from endoscopic mucosal resection. In the two photos, evidence of malignant change is apparent in the left half. Bar, 500 μm. *C*, a nonmalignant adenomatous colorectal polyp obtained by endoscopic mucosal resection. Adenomatous change is apparent at the edge of the colorectal mucosa. Bar, 500 μm.

(29). Therefore, these double-negative feedback loops operating through the miR-21 promoter may contribute to the self-reinforced expression of miR-21. This feedback could further lead to the mutually exclusive expression patterns observed between miR-21 RNA and PDCD4 protein in colorectal cancer. It also remains to be determined which transcriptional factors that are normally inhibited by PDCD4 are crucial for *miR-21* gene induction in colorectal cancer. The molecular mechanisms involved in functional suppression of these transcription factors by PDCD4 could significantly advance our understanding of cancer progression. Therefore, elucidation of the molecular mechanisms involved in the entire regulatory network formed by miR-21 is important for understanding the initial stages of colorectal carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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