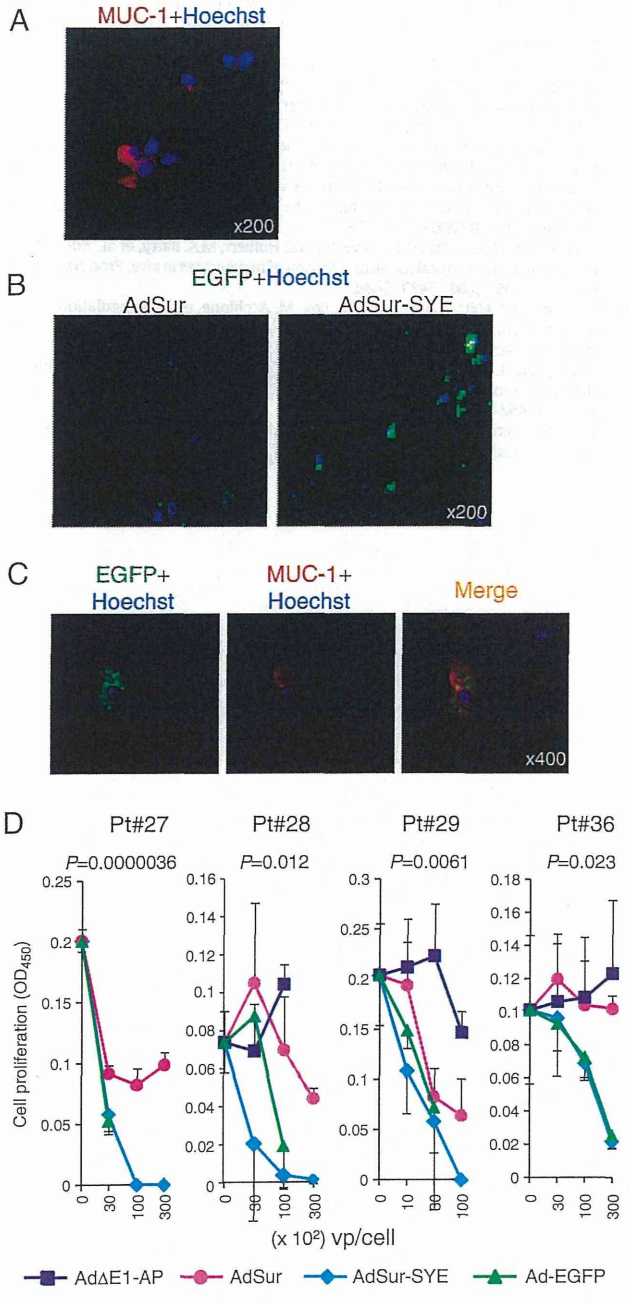

Fig. 6. Oncolytic activity of AdSur-SYE in human pancreatic cancer tissues. A) Co-culture of pancreatic cancer cells with MEFs. The pancreatic cancer cells were co-cultured with MEFs for 6 days. After the fixation with 4% paraformaldehyde, the cells were stained by monoclonal mouse α-human MUC-1 antibody (E29) followed by the 2nd antibody of Alexa Fluor 647 and hoechst33258. B) $EGFP^+$ cells in pancreatic cancer cells infected with viruses. The tumor cells co-cultured with MEFs were infected with viruses. The photographs were representatives of cells infected with AdSur-SYE and AdSur at 1×10^3 vp/cell at day 6, which were assessed by ArrayScan VTI HCS Reader. C) $EGFP^+MUC\text{-}1^+$ pancreatic cancer cells. The photographs are representatives of $EGFP^+$ and $MUC\text{-}1^+$ pancreatic cancer cells infected with AdSur-SYE at 1×10^3 vp/cell at day 6 by ArrayScan VTI HCS Reader. D) Cytotoxic activity of adenoviruses in pancreatic cancer cells co-cultured with MEFs. Tumor cells prepared from 4 surgical specimens of pancreatic cancer were co-cultured with MEFs and the cells were infected with adenoviruses. Six days after the infection, cell numbers were measured by an *in vitro* cell growth assay. The differences between the OD_{450} values of total pancreatic cancer cells and MEFs and those of MEFs alone were presented. The assays were carried out in 4 wells, and the mean ± standard deviation was plotted. P value: AdSur-infected cells versus AdSur-SYE-infected cells at 1×10^4 vp/cell.

suppress a liver transduction may effectively allow for the development of vectors to specifically transduce certain tumors even through systemic administration [28].

Pancreatic cancer is one of the most intractable cancers, and new therapeutic approaches that can effectively target its spread *in vivo* are urgently needed. In pancreatic cancer, a regional therapy is also particularly relevant, because locally advanced cases, which are surgically unresectable but can be accessible by ultrasound- or CT-guided percutaneous injection or endoscopic ultrasound-guided injection, require a strong local control strategy [1,5]. The selection of patients, whose cancer tissues are suitable to this targeting strategy, is one of the most important issues to achieve sure success in clinical trials. Since, in this study, the infection of AdSur-SYE is easily detected in the sliced tissues by EGFP expression under fluorescence microscopy (Fig. 5C), the selection of patients on biopsy samples may be feasible in the clinical setting. Although there is no metastasis in the locally advanced cancer at the time of diagnosis, distant metastases frequently appear in the process of disease progression. As a next step, a combination with other approaches such as immune therapies and systemic chemotherapies may contribute to mounting a systemic antitumor effect against pancreatic cancer.

In conclusion, we showed that a survivin promoter-regulated oncolytic adenovirus, which displays a pancreatic cancer-targeting ligand, resulted in an increased infectivity and stronger oncolytic potency than an untargeted adenovirus in pancreatic cancer tissues did. CRAds in combination with a targeting strategy are a promising candidate for the next generation of oncolytic virotherapy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2014.07.053.

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A human cancer xenograft model utilizing normal pancreatic duct epithelial cells conditionally transformed with defined oncogenes

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Pancreatic ductal adenocarcinomas (PDACs) are considered to arise through neoplastic transformation of human pancreatic duct epithelial cells (HPDECs). In order to evaluate the biological significance of genetic and epigenetic alterations in PDACs, we isolated primary HPDECs and established an *in vitro* carcinogenesis model. Firstly, lentivirus-mediated transduction of KRAS^{G12V}, MYC and human papillomavirus 16 (HPV16) E6/E7 under the control of a tetracyclin-inducible promoter efficiently immortalized and transformed primary HPDECs, which gave rise to adenocarcinomas subcutaneously in an immune-deficient mouse xenograft model, depending on expression of the four genes. The tumors regressed promptly upon shutting-off the oncogenes, and the remaining tissues showed histological features corresponding to normal ductal structures with simple columnar epithelium. Reexpression of the oncogenes resulted in development of multiple PDACs through pancreatic intraepithelial neoplasia-like structures. We also succeeded in efficient immortalization of primary HPDECs with transduction of mutant CDK4, cyclin D1 and TERT. The cells maintained a normal diploid status and formed duct-like structures in a three-dimensional culture. In combination with p53 silencing, KRAS^{G12V} alone was sufficient to fully transform the immortalized HPDECs, and MYC markedly accelerated the development of tumors. Our PDAC model supports critical roles of KRAS mutations, inactivation of the p53 and p16-pRB pathways, active telomerase and MYC expression in pancreatic carcinogenesis and thus recapitulates many features of human PDAC development. The present system with reversible control of oncogene expression enabled *de novo* development of PDAC from quasinormal human tissues preformed subcutaneously in mice and might be applicable to carcinogenesis models in many organ sites.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human cancers. It is characterized by late diagnosis due to lack of early symptoms, extensive metastasis and high resistance to chemotherapy and radiation. Despite advances in the clinical management of the disease, only ~6% of patients survive 5 years after diagnosis and prognosis of PDAC remains poor (1–4). Further work is needed to elucidate the events driving pancreatic carcinogenesis to have any hope of eventually conquering this disease.

Although the cells-of-origin for PDAC remain controversial, a multistep carcinogenesis process has become widely accepted. In this concept, PDAC arises from precursor lesions termed Pancreatic Intraepithelial Neoplasia (PanIN) involving multiple genetic alterations. Four genes, KRAS (>90%), p16/CDKN2A (>95%), p53 (50–75%) and DPC4/SMAD4 (55%) are found to be commonly mutated or inactivated in PDAC (1–3). These are classified as driver alterations for development of PDAC.

Genetically engineered mouse models of PDAC are excellent experimental systems which have helped to improve our understanding of the disease. In these models, invasive cancers that resemble human PDAC can be induced by combinations of two or three genetic alterations in precursors of mouse pancreas (5). However, there are biological differences between mice and humans. Since the altered expression of the genes is sufficient to produce metastatic cancers, limits exist as to the capacity to investigate new candidate cancer gene functions. Therefore, other approaches using human materials, such as human pancreatic cancer cell lines and human normal pancreatic cells, are also needed, in addition to the use of genetically engineered mouse models.

Recently, we have established *in vitro* multistep carcinogenesis models for cervical cancer, epithelial ovarian cancer and oral cancer (6–8). In the present study, taking advantage of this background, we could successfully immortalize and transform primary human pancreatic duct epithelial cells with defined genetic elements. Using a tetracyclin-inducible system, we found, for the first time, that bimodal expression of oncogenes brings out reversible features of transformation and recurrence of multiple PDACs through PanIN-like structures. Unique features and applications of this approach are discussed.

Materials and methods

Isolation of human pancreatic duct epithelial cells

Normal human pancreatic tissues were obtained with written consent from patients who underwent abdominal surgery for a pancreaticobiliary diseases. Human pancreatic duct epithelial cell (HPDEC4) was obtained from pancreas tail of a patient who underwent resection for pancreatic head carcinoma. No cancerous lesions near the specimen were confirmed by histopathological examination. HPDEC6 and HPDEC 7–11 were obtained from patients who underwent resection for solid pseudopapillary neoplasm and ampullary cancer, respectively. The pancreas tissue was confirmed macroscopically normal with no pathological lesions by pathologists, minced into pieces suspended in Hanks' balanced salt solution with 1 mg/ml Liberase HI (Roche) and incubated at 37°C for 60 min. The dissociated tissues were subsequently filtered with a 70 μm pores Cell Strainer (BD Biosciences) and isolated primary epithelial cells were seeded on type I collagen-coated dishes (Corning). HPDECs were maintained in serum-free keratinocyte serum-free medium (Invitrogen) supplemented with 5 ng/ml epidermal growth factor (Sigma), 50 μg/ml of bovine pituitary extract (Hammond CELL TECH), 10% fetal bovine serum, 2 mM NAC (N-Acetyl-L-cysteine; Wako), 0.2 mM Asc-2P (L-ascorbic acid 2-phosphate; Wako), which is similar to the K-NAC medium described earlier (9), but further supplemented with 5 μM Y-27632 (Selleck Chemicals).

Pancreatic cancer cell lines

Pancreatic cancer cell lines, AsPC-1, BxPC-3, Capan-1, Capan-2, HPAC, PANC-1 and MIAPaCa-1, were obtained from ATCC and grown in Dulbecco's modified Eagle's medium (DMEM; Nacalai tesque) containing 10% fetal bovine serum. These cell lines have not been authenticated by the authors.

Vector construction and retroviral infection

Oncogenic KRAS (KRAS^{G12V}) and a mutant form of MYC (MYC^{T58A}) generated by site-directed mutagenesis were cloned and recombined into retroviral expression vectors to generate pCLXSN-KRAS^{G12V}and pCMSCVpuro-MYC^{T58A}, as described previously (8). A retroviral vector pCLSI-MSCVhyg-H1R-p53shRNA was designed to express a p53-specific short

Abbreviations: DOX, doxycyclin; HPDEC, human pancreatic duct epithelial cell; HPV16, human papillomavirus 16; PanIN, Pancreatic Intraepithelial Neoplasia; shRNA, short hairpin RNA.

hairpin RNA (shRNA) targeting 5′-GACTCCAGTGGTAATCTAC-3′ (10). Lentiviral vectors expressing TERT, cyclin D1 and mutant CDK4 (CDK4^{R24C}: an inhibitor resistant form of CDK4) were constructed by recombination with a lentiviral vector, CSII-CMV-RfA as described previously (8). CSII-TRE-Tight-RfA was generated by replacing the elongation factor promoter in CSII-EF-RfA with the tetracycline-responsive promoter from pTRE-Tight (Clontech). CSII-TRE-Tight-16E6E7-2A-MYC^{T58A}-2A-HRAS^{G12V}(-KRAS^{G12V}) was constructed by inserting 16E6E7, MYC^{T58A} and HRAS^{G12V} (KRAS^{G12V}) segments separated by sequences encoding the autonomous 'self-cleaving' 2A peptides derived from foot-and-mouse disease virus into CSII-TRE-Tight-RfA. The production of recombinant viruses was as detailed earlier (8,11).

Ethics statement

Tissues from patients: Normal human pancreatic tissues were obtained with written consent from patients. The study was approved by the National Cancer Center Institutional Review Board. Animal study: Animal studies were carried out according to the Guideline for Animal Experiments in National Cancer Center, which meet the ethical standards required by the law and the guidelines about experimental animals in Japan, and approved by the Committee for Ethics in Animal Experimentation of the National Cancer Center. Cell line information: Pancreatic cancer cell lines, AsPC-1, BxPC-3, Capan-1, Capan-2, HPAC, PANC-1 and MIAPaCa-1, were directly obtained from ATCC. Among them AsPC-1, BxPC-3, PANC-1 and MIAPaCa-1 were purchased in 1993, and AsPC-1, BxPC-3, PANC-1 and MIAPaCa-1 were purchased in 2009. They have been passaged and used in our laboratory for <6 months after resuscitation though these cell lines have not been authenticated by the authors.

Western analysis

Western blotting was conducted as described previously (8). Antibodies used were listed in the Supplementary Materials and methods, available at *Carcinogenesis* Online.

G-banding karyotyping analysis

The karyotype analysis was carried out using standard G-banding by an outsourced service (Mitsubishi Chemical Medience).

Three-dimension culture for HPDECs

The three-dimensional culture protocol was a modification of the procedure described previously (12). Briefly, cells were embedded in type I collagen (final 2 mg/ml solution, AteloCell IPC; Koken) alone or in a mixture of type I collagen (final 2 mg/ml) and Matrigel (final 2 mg/ml, 354248; BD Biosciences) mixed with growth medium containing 10 mM HEPES. Cells were cultivated in the gel on a cell culture insert (353494, FALCON) dipped in growth medium for 3–4 weeks. For histological analysis, cultures were fixed with 10% neutral buffered Formalin (4% formaldehyde) and embedded in paraffin.

Immunohistochemical examination

Formalin-fixed and paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated through a graded ethanol series (100–70%). For antigen retrieval, slides were immersed in a citrate buffer (pH 6.4) or Target Retrieval Solution (pH9) (S2367; Dako). The slides were then incubated in methanol containing 0.3% H_2O_2 to inhibit endogenous peroxidase activity. After washing, primary antibodies were applied for 1 h and binding was detected using an Envision Kit (Dako). Color development was achieved with 3, 3′-diaminobenzidine as chromogen and hematoxylin counterstaining.

Tumorigenesis in Nude Mice

All surgical procedures and care of the animals were in accordance with institutional guidelines. A 100 µl volume of 10^6 cells in a 1:1 mixture of Matrigel (354248; BD Biosciences) was subcutaneously injected into female BALB/c nude mice (Clea Japan). Doxycyclin (DOX) was given to the recipient mice via their drinking water (1 mg/ml in 5% sucrose). Tumor size was measured every other day using calipers and tumor volume was estimated using the following formula: $V = (LW^2)/2$, where V, volume (mm^3); L, largest diameter (mm) and W, smallest diameter (mm).

Results

Conditional tumorigenic transformation of primary HPDECs with human papillomavirus type 16 E6/E7, MYC and oncogenic RAS^{G12V}

Previously, we demonstrated that introduction of human papillomavirus type 16 (HPV 16) E6 and E7 genes (E6E7), oncogenic HRAS (HRAS^{G12V}) and MYC (MYC^{T58A}) is sufficient for tumorigenic transformation of normal human cells, such as cervical keratinocytes, tongue keratinocytes and bronchial epithelial cells (6–8). Here, a single polycistronic vector designed to express E6E7, MYC and KRAS^{G12V} (EMR) under control of a tetracycline-responsive promoter was transduced into a strain of primary HPDEC6 together with the tetracyclin-dependent transcriptional activator (tetOFF) (Figure 1A). DOX-dependent silencing of the oncogenes was confirmed by western blotting. The growth of HPDEC6 cells depended on expression of the transgenes (Figure 1B and C). When transduced cells were subcutaneously transplanted into nude mice, they formed tumors within 1 month (Table I, Figure 1D). Most parts of the resultant tumors showed histological features corresponding to moderately to poorly differentiated adenocarcinoma (Figure 1E, left panel) and some parts of periphery of the tumors showed those corresponding to well-differentiated adenocarcinoma (Figure 1E, middle panel). When the tumors became evident, DOX was administered to animals and this resulted in halted tumor growth, followed by almost complete regression. Close examination of the remaining tissues showed ductal structures consisting of simple columnar epithelium (Figure 1E, right panel). The epithelial cells derived from HPDEC maintained quasinormal duct structures for at least 1 month in subcutaneous sites of mice without any sign of tumor growth.

After confirmation of regression of initial tumors, DOX was withdrawn to examine whether tumors regrew or not. About 2 months after the withdrawal, overt tumors reappeared at the sites of initial tumors. Interestingly, a mixed histology composed of simple columnar cells and malignant cells was observed in the reformed tumors (Supplementary Figure 1C, available at *Carcinogenesis* Online).

In a genetic engineered tumor model of *Rattus* pancreas, expression of oncogenic *Kras* or *Hras* induces pancreatic cancer, whereas in human lesions, oncogenic KRAS mutations are most common (13). In our xenograft model, HRAS^{G12V} showed essentially the same results as obtained with KRAS^{G12V} (Figure 1D and E; Supplementary Figure 1A, available at *Carcinogenesis* Online). We confirmed the tumorigenic effects of the oncogenes by employing another independent batch of HPDEC derived from other patients (Supplementary Figure 1B, available at *Carcinogenesis* Online). These results support that oncogenic RAS signaling plays a crucial role in pancreatic cancer development.

Temporal analyses of PDAC development from quasinormal tissues derived from HPDEC

To more precisely characterize the histological features of the tumors and to analyze the temporal development of tumors from quasinormal ductal structures, tumors were collected and examined at different time points before and after DOX administration, and after withdrawal of DOX.

As in the pilot experiment described above, initial tumors appeared within 1 month, and the tumor volume exceeded 300 mm^3 within 20 days, when DOX was administered. After the initial tumors regressed to masses <150 mm^3, DOX was withdrawn. Reproducibility of not only the initial tumor growth but also the regression and regrowth of the tumors was excellent, judging from the standard error bars of the tumor sizes of individual animals and even tumor sizes for different animals (Figure 2A). The initial tumors were confirmed to exhibit moderately to poorly differentiated adenocarcinoma histology (Figure 2B). Immunohistochemical analyses indicated that the tumors expressed MUC1 and MUC5AC, which are also widely expressed in human PDAC clinical samples. On the other hand, expression of CDX-2 and MUC6 was not detected (Figure 2C). Thus, the initial tumors derived from HPDEC6-*tre* EMR cells resemble human PDACs in terms of microscopic histology and immunohistochemical expression pattern.

Subsequently, we examined regulation of transgenes *in vivo* by immunohistochemical detection of MYC protein. The initial tumors which should express EMR showed strong staining of MYC, whereas no expression was detected in the epithelial cells in the tissues remaining after DOX administration for 60 days. Staining for Ki67 indicated that repression of the oncogenes reduced cell proliferation (Figure 2B). These results confirmed that regulation of oncogene expression also functioned *in vivo* as well as *in vitro*.

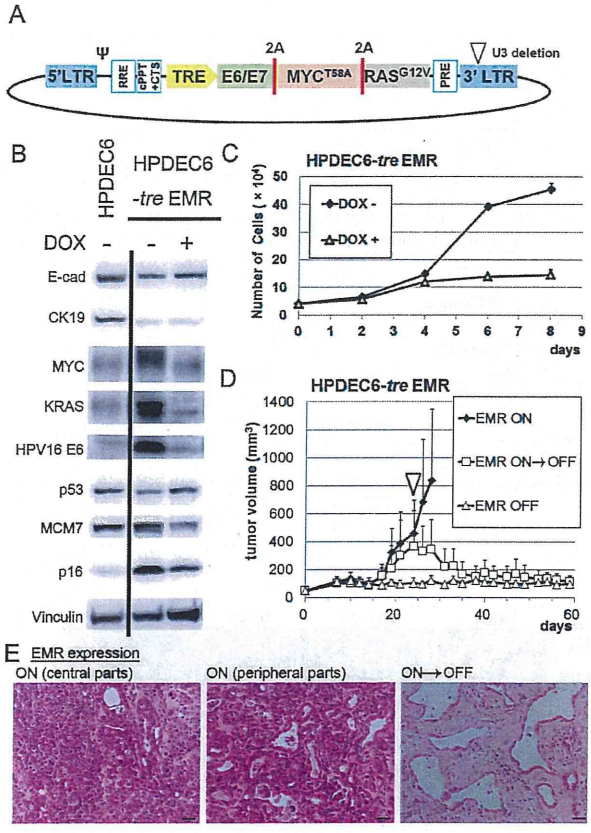

Fig. 1. Expression of HPV16 E6E7, KRAS^{G12V} and MYC^{T58A} is sufficient for full transformation of primary human pancreatic duct cells. (**A**) Schematic illustration of a single polycistronic lentiviral vector in which expression of E6E7, MYC^{T58A} and KRAS^{G12V} (EMR) is regulated by DOX (tetOFF). TRE, tetracyclin-responsive elements; 2A, 'self-cleaving' 2A peptides derived from foot-and-mouse disease virus, ψ, packaging signal regions; RRE, rev responsive element; cPPT, central polypurine tract; CTS, central termination sequence; PRE, woodchuck hepatitis virus posttranscriptional regulatory element. (**B**) Expression of the transgene products and several target proteins possibly regulated by them was determined by western blotting. Lane 1, parental HPDEC6 cells at passage 8. Lanes 2 and 3, HPDEC6 cells transduced with EMR and tetOFF at passage 9 were propagated and treated with 1 μg/ml DOX (DOX+) or vehicle (DOX–) for 5 days at passage 16. (**C**) Growth curves of HPDEC6-*tre* EMR cells with or without DOX. Cells were seeded at a density of 4×10^4 cells per 22 mm dish (BD Biosciences) at day 0 and counted at the indicated days. DOX (1 μg/ml) or vehicle (70% ethanol) was added from day 2 and thereafter every other day with fresh medium. Each point is the mean of the triplicates ± standard deviation. (**D**) Tumor-forming ability of HPDEC6-*tre* EMR. Cells were subcutaneously injected into nude mice (1×10^6 cells) and tumor size was measured every other day. One mouse was fed with drinking water supplemented with 1 mg/ml DOX from day 0 (open triangles). Of the other two mice, one mouse was administered with DOX from day 24 (white arrowhead) when the mean tumor volume exceeded 300 mm^3 (open rectangles). Each point is the mean of the tumor volume ± standard deviation ($n = 6$). (**E**) Histopathology of the xenografts stained with hematoxylin and eosin. A representative image of central parts of the tumors, which corresponds to moderately to poorly differentiated carcinoma (left panel), and that of peripheral parts of the tumors, which corresponds to well-differentiated adenocarcinoma from the mouse without DOX administration (closed rhombuses in D) killed at day 28. Right panel shows a representative image of the remaining tissue from the mouse after tumor regression by DOX administration (open squares in D) killed at day 60. Scale bars, 50 μm.

MYC reexpression was detected at as early as 6 days after DOX withdrawal and significant increase of Ki-67 positive cells was detected at day 12, when overt tumor growth was observed. Mixed histology composed of simple columnar epithelium, well-differentiated adenocarcinomas and undifferentiated carcinomas was observed at day 21. The population of undifferentiated cells showed strong staining of MYC and Ki67 was present, whereas staining of CK19 and MUC1 was very weak (Figure 2D). In contrast, the cells of simple epithelium were negative for MYC and Ki67 and positive for CK19 and/or MUC1. Intriguingly, the cells used for transplantation were near diploid, and 45% (9/20) were normal diploid (Supplementary Table 1, available at *Carcinogenesis* Online), although it has been reported that HPV16 E6 and E7 readily induce chromosomal instability and tetraploidy (14–16). Taken together, these results suggest that E6E7, oncogenic RAS and MYC are sufficient for tumorigenic transformation of primary HPDECs without further genetic alterations.

Immortalization of normal HPDECs

We could conditionally immortalize and transform primary HPDECs with the four oncogenes and observe transition of quasinormal HPDEC-derived duct-like structures to PanIN-like and PDAC-like histological structures in mouse xenografts. However, this model does not faithfully mimic pancreatic carcinogenesis since four oncogenes were expressed at the same time and HPV is not considered to be at all involved in human PDAC development. To analyze events of PDAC development, normal HPDECs should be freely cultivated *in vitro*. However, no normal HPDEC lines have been available except for a HPDEC line immortalized with HPV16 E6 and E7, and reliable methods for long-term culture of HPDECs have been lacking (9). As we recently found that a ROCK inhibitor, Y27632, could inhibit keratinocyte differentiation through a novel NOTCH-ROCK pathway (17), we applied it to the reported medium for HPDEC culture. With the modified method, plating efficiencies of HPDECs were remarkably improved, but they eventually stopped growing after 9–38 population doublings depending on cell batches (Figure 3A and B). As senescence of normal human epithelial cells in regular culture conditions is caused by activation of RB and telomere shorting, we tried to immortalize the cells by activating telomerase through transduction of TERT, and by inactivating the pRB pathway with mutant CDK4 (CDK4^{R24C}, an inhibitor resistant form of CDK4) and cyclin D1, into primary HPDECs with lentiviral vectors (18). With this strategy, we could immortalize pooled populations of HPDEC-4, -6, -8 and -10. The combination of CDK4^{R24C}, cyclin D1 and TERT (K4DT) clearly extended the life span over 40 population doublings and virtually immortalized HPDEC10 cells, whereas TERT alone hardly extend the life span (Figure 3A). Expression of the transgenes was confirmed by immunoblotting (Figure 3B). The immortalized HPDECs, as well as the primary cells, expressed cytokeratin 19 and E-cadherin (Figure 3C). Among them karyotype of HPDEC4-K4DT, HPDEC8-K4DT and HPDEC10-K4DT cells were analyzed and revealed that the majority of the cells were normal diploid (Supplementary Table 1, available at *Carcinogenesis* Online), indicating this is an efficient strategy to immortalize normal HPDECs with minimal alteration of the genetic background.

In three-dimensional culture with type I collagen as the extracellular matrix, HPDEC4-K4DT cells could form duct-like structures in the gel (Figure 3D), suggesting retention of a biological characteristic of HPDECs after immortalization. This could be a good tool to examine early carcinogenic events.

Tumorigenic transformation of HPDEC4-K4DT cells with transduction of p53-specific shRNA, KRAS^{G12V} and MYC

Since HPDEC4-K4DT cells were pooled populations established from primary culture of pancreatic tissue, proliferation of mesenchymal cells sometimes compromised that of epithelial cells, although the majority of HPDEC4-K4DT cells appeared to be epithelial in nature. To remove mesenchymal cells, we cloned epithelial cell lines by limiting dilution. Then, we examined the effects of several oncogenes relevant to pancreatic carcinogenesis on the HPDEC4-B7 cell line, a purified epithelial cell line with normal diploidy. Since, the pRB pathway was already inactivated and telomerase activated in the cells, we tested three genetic alterations; inactivation of p53 using shRNA,

Table I. Summary of xenotransplantation of HPDECs

Cells	Number of tumors per subcutaneous injection
HPDEC6	
-*tre* E6E7-MYC^{T58A}-KRAS^{G12V}	6/6 (28) [exp 1; Figure 1] [a]5/6 (26) [exp 2; Figure 2]
-*tre* E6E7-MYC^{T58A}-HRAS^{G12V}	6/6 (28) [Supplementary Figure 1, available at *Carcinogenesis* Online)]
HPDEC4	
-*tre* E6E7-MYC^{T58A}-HRAS^{G12V}	6/6 (3) [Supplementary Figure 1, available at *Carcinogenesis* Online] 4/6 (59) [Supplementary Figure 1, available at *Carcinogenesis* Online; reexpression)]
HPDEC4-K4DT (B7)	
-p53 shRNA-KRAS^{G12V}-MYC^{T58A}	6/6 (48) [Figure 4]
-p53 shRNA-KRAS^{G12V}-vector	4/6 (98) [Figure 4]
-p53 shRNA- MYC$^{T58A\text{-vector}}$	0/6 (100)
-Luc shRNA-KRAS^{G12V}-MYC^{T58A}	0/6 (100)
-p53 shRNA-vector-vector	0/6 (100)

Numbers in parentheses indicate observation time points (days) when mice were killed.
[a]Note that one of the six tumors was less than the criterial volume (150 mm^3), but it exhibited adenocarinoma-like histology. Words in square brackets are information of experiment number and related figure.

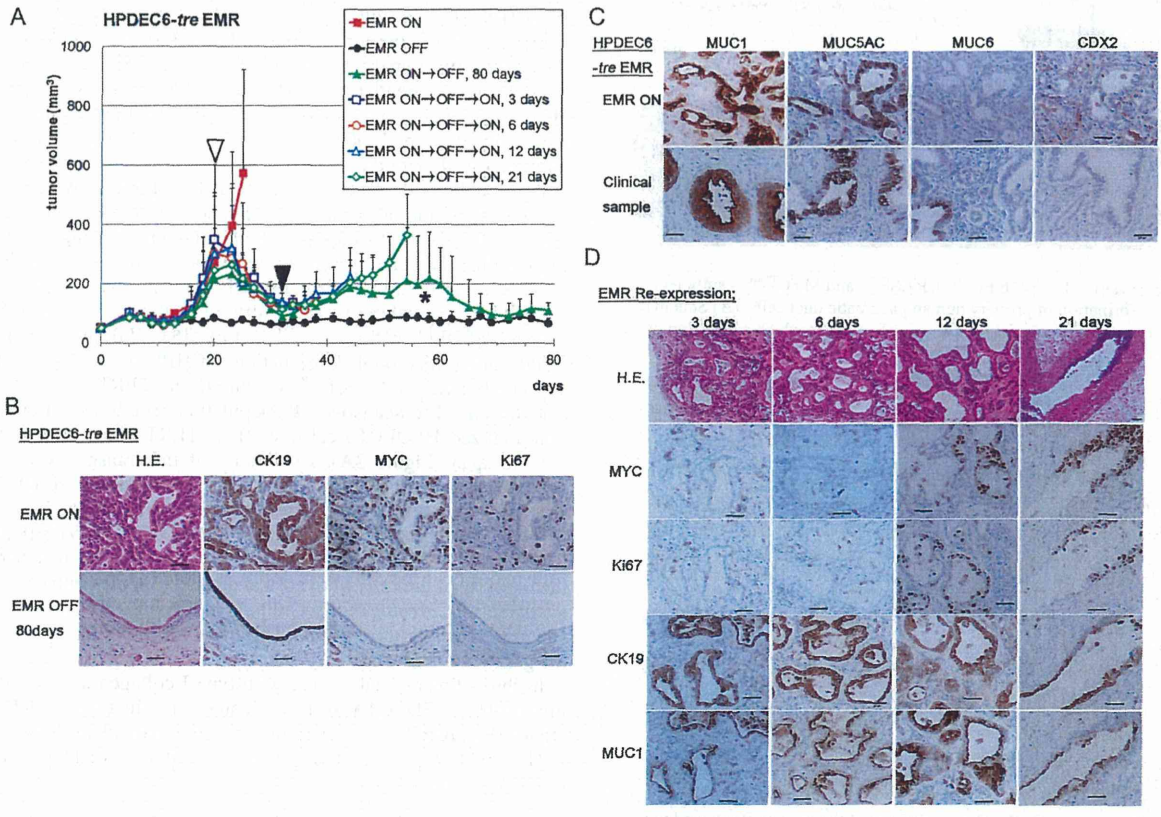

Fig. 2. Development of PDAC-like tumors from quasinormal tissues derived from *HPDEC6-tre* EMR cells. (**A**) Growth curves of the tumors. *HPDEC6-tre* EMR cells were subcutaneously injected into nude mice (1×10^6 cells). One mouse was administered with DOX from day 0 as described in Figure 1D (black closed circles). Among the other six mice, one mouse was killed at day 26 as mean tumor volume exceeded 500 mm^3 (red closed squares) and five mice were administered with DOX from day 20 (white arrowhead) until day 31 when all the tumors regressed almost completely. Then DOX administration to four mice was discontinued at day 32 (black arrowhead), and the mice were killed at different time points as indicated, namely 3 days (purple open squares), 6 days (orange open circles), 12 days (blue open triangles) and 21 days (green open rhombuses) after the DOX withdrawal. The other mouse was continuously administered with DOX until day 80 (green closed triangles). *Note that the apparent increase of the tumor volume around day 40–60 in this mouse was due to cyst-like soft mass formation but not solid tumors. Each point is the mean of the tumor volume ± standard deviation ($n = 6$). (**B**) Histopathology of subcutaneous tumors of the mouse killed at day 26 and remaining tissues after tumor regression in the mouse killed at day 80. (**C**) Immunohistochemical comparison of the PDAC-like tumors and a typical human PDAC. Expression of MUC1, MUC5AC, MUC6 and CDX2 were examined. Note that positive signals in the xenograft by CDX2 staining were localized at the positions where Matrigel remained. (**D**) Development of PDAC-like tumors from the quasinormal pancreatic duct-like structures subcutaneously formed in mice. Temporal development of tumors were analyzed by hematoxylin and eosin staining and immunohistochemical staining of MYC, Ki67, CK19 and MUC1. Scale bars represent 50 μm.

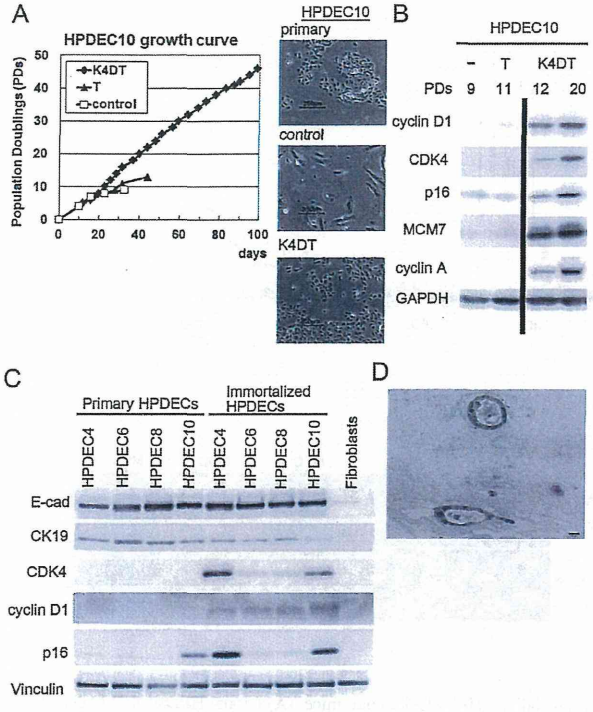

Fig. 3. Immortalization of primary HPDECs. (**A**) Growth curves of HPDEC10 cells transduced by indicated genes at passage 0. The cells transduced with mutant CDK4, cyclin D1 and TERT (K4DT) were virtually immortalized and maintained epithelial cell morphology without growth retardation, whereas TERT-transduced cells (T) and control cells senesced around population doublings 10. Scale bars, 250 μm. (**B**) Expression of CDK4 and cyclin D1 as well as some cell cycle-associated genes were examined by western blotting. (**C**) Expression of the transgenes and duct epithelial markers in four batches of primary HPDECs and those immortalized by transduction of K4DT were confirmed by western blotting. Fibroblasts derived from the same pancreatic tissue from which HPDEC4 cells were obtained were used as a control. (**D**) HPDEC4-K4DT cells were cultivated in a collagen gel for 4 weeks and fixed, paraffin embedded, sectioned and stained with hematoxylin and eosin. A scale bar represents 20 μm.

introduction of oncogenic KRAS and altered expression of MYC. Combinations of p53-specific shRNA (p53shRNA), $KRAS^{G12V}$ and MYC were serially transduced into HPDEC4-B7 cells. Expression of KRAS, MYC and downregulation of p53 were confirmed by western blotting. The expression levels of transgenes were comparable with those of pancreatic cancer cell lines (Figure 4A).

In three-dimensional culture, p53 shRNA-expressing HPDEC4-B7 cells formed larger duct-like structures than control shRNA-expressing cells, which formed similar duct-like structures as the parental pooled HPDEC4-K4DT cells (Figure 4B). In accordance with the larger duct-like structures formed by HPDEC4-B7-p53shRNA cells, most showed Ki67 staining. To examine effects of $KRAS^{G12V}$ expression, we compared HPDEC4-B7-p53shRNA and HPDEC4-B7-p53shRNA-$KRAS^{G12V}$ cells, since oncogenic RAS might further induce p53 expression (19). With $KRAS^{G12V}$ expression, PanIN-like structures were recapitulated and most of p53shRNA-$KRAS^{G12V}$ cells were positive for Ki-67 staining (Figure 4B and C). In this system, the cells already express $KRAS^{G12V}$ before being embedded in the gel, which is not the real situation for early events in carcinogenesis. So we also tried conditional $KRAS^{G12V}$ expression by DOX in 3D culture, but failed to observe formation of PanIN-like structures, although induction of $KRAS^{G12V}$ did support faster proliferation of cells in monolayer culture (Supplementary Figure 2, available at *Carcinogenesis* Online).

To examine tumorigenic ability, 1×10^6 cells were injected subcutaneously into nude mice. HPDEC4-B7-p53shRNA-$KRAS^{G12V}$-MYC^{T58A}-expressing cells formed tumors within 2 months, whereas p53shRNA- and $KRAS^{G12V}$-expressing cells formed tumors only after a long latent period (Figure 4D). Tumors derived from HPDEC4-B7 cells histologically were poorly differentiated adenocarcinomas and expressed MUC1/MUC5AC as HPDEC6-*tre* EMR cells did (Figure 4E). The other combinations of the transgenes did not confer tumorigenicity on the cells (Figure 4D, Table I).

Notably, 40% (8/20) of HPDEC4-B7-p53shRNA-$KRAS^{G12V}$-MYC^{T58A} cells were normal diploid (Supplementary Table 1, available at *Carcinogenesis* Online). These results again indicate that KRAS and MYC on a background of inactivation of pRB and p53 pathways and active telomerase are sufficient for tumorigenic transformation of normal HPDECs without further genetic alterations.

Discussion

Establishment and *in vitro* culture of normal human pancreatic cells is important not only for determination of genetic alterations required for malignant transformation but also as ideal controls for identifying therapeutic targets specific for cancer cells. However, *in vitro* cultures of normal human pancreatic cells are very limited. Thus, most *in vitro* models for human PDACs have utilized established cancer cell lines. Nevertheless, there are a few human pancreatic epithelial cell lines immortalized with HPV16 E6 and E7 or ectopic expression of TERT (9,20). It has been shown that HPV16 E6 and E7, oncogenic $Kras^{G12D}$ and SV40 small t antigen are sufficient to transform TERT-immortalized HPDECs, and oncogenic KRAS alone can transform human pancreatic epithelial cells preimmortalized with HPV16 E6 and E7 (20,21). In the present report, we document that HPV16 E6 and E7, oncogenic RAS and MYC are sufficient to transform primary normal HPDECs. Furthermore, oncogenic RAS and inactivation of p53 are sufficient to transform HPDECs immortalized with $CDK4^{R24C}$, cyclin D1 and TERT. All these results suggest that the number of genetic alterations required for malignant transformation is much smaller than the average of 48 alterations detected by global direct sequencing (22,23).

Numerous genetically engineered mouse models mimicking PanINs, intraductal papillary mucinous neoplasm, mucinous cystic neoplasms and PDACs have been successfully developed in recent years (5,24–27). However, *in vitro* cultures of the human counterparts are not available. No study has succeeded in recapitulating precancerous lesions *ex vivo*. Better understanding of the genetics of precancerous lesions is essential for their early detection and prophylaxis. In this study, we succeeded in reconstructing ductal structures consisting of simple columnar epithelium with primary HPDECs, conditionally expressing four oncogenes sufficient for malignant transformation. From the quasinormal duct structures, development of PDACs through PanIN-like structures could be observed. Our methodology described in here could be applied to many other cell types, since we have demonstrated that HPV16 E6 and E7, oncogenic RAS and MYC are sufficient to transform different types of normal human cells (6,8,28).

Four genes, KRAS, p16/CDKN2A, p53 and DPC4/SMAD4 are most commonly mutated or inactivated in PDACs. These are classified as driver alterations for PDAC development. In the majority of not only PDACs but also many other cancers, telomerase is activated and overexpression of MYC is observed. The tumors in this report presented some typical features of PDAC, even when etiologically irrelevant HPV16 oncogenes were used for transformation (29). This might not be surprising since the viral oncogenes can mimic frequent alterations found in PDACs, namely E6 targets p53 for degradation and activates telomerase and E7 binds and inactivates pRB. Our tumorigenic cells harbored normal diploid or near diploid chromosomes, suggesting combinations of these transgenes are sufficient for malignant transformation without further genetic alteration. The experimental results allow us to conclude that the mechanisms of how the two major tumor suppressor pathways are inactivated and the

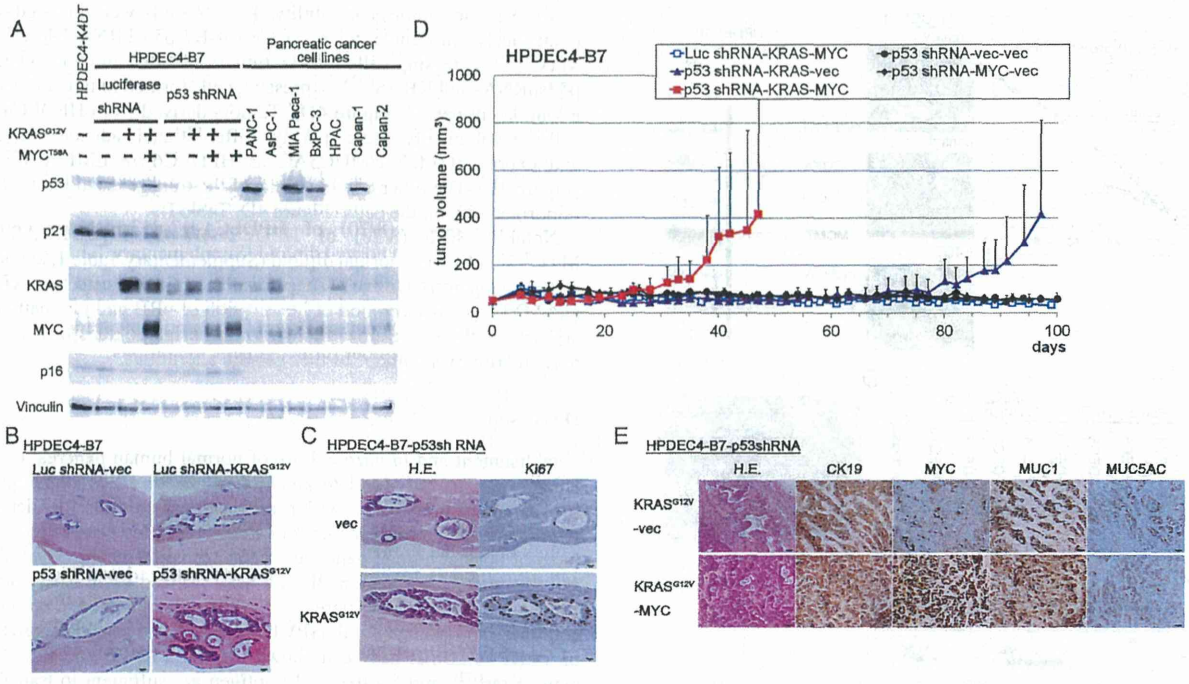

Fig. 4. Transduction of p53 shRNA, KRAS^{G12V} and MYC^{T58A} confers tumorigenicity to immortalized HPDECs in nude mice. (**A**) Clonal HPDEC4-B7 cells established from HPDEC4-K4DT cells were serially infected with p53 shRNA-, KRAS^{G12V}- and MYC^{T58A}-expressing retroviruses. Expression of KRAS, MYC, p53 as well as p21 and p16 was examined in comparison with those in several pancreatic cancer cell lines by western blotting. (**B** and **C**) HPDEC4-B7 cell lines expressing indicated transgenes were cultivated in a mixture of collagen type I and Matrigel. Formation of duct-like structures were analyzed as described in Figure 3D. (**B**) HPDEC4-B7-Luc shRNA-vec cells reconstituted duct-like structures in 3 weeks (upper left panel). The addition of p53 shRNA contributed to formation of large duct structures (lower left panel). Expression of KRAS^{G12V} and p53 shRNA induced dysplastic epithelial structures similar to PanIN, whereas KRAS^{G12V} expression alone did not (right panels). Scale bars represent 20 μm. (**C**) The PanIN-like structures formed by HPDEC4-B7-p53shRNA-KRAS^{G12V} cells were stained for Ki67. Hematoxylin and eosin (H.E.) staining (left panels) and Ki67 staining (right panels) are shown. Note that most of the cells were positive for Ki-67 staining, whereas the cells expressing p53-shRNA alone were not. Scale bars represent 20 μm. (D) Tumor growth curves of the HPDEC4-B7 cell lines with indicated transgenes. Cells were subcutaneously injected into nude mice (10^6 cells). The mouse transplanted with HPDEC4-B7-p53shRNA-KRAS^{G12V}-MYC^{T58A} was killed at 47 days (red squares) when tumors arose from all the six injected sites, whereas that with HPDEC4-B7-p53shRNA and KRAS^{G12V} was killed at 97 days (purple triangles) when large tumors grew from four out of six injected sites. The other cells did not show tumorigenicity within 3 months. Each point is the mean of the tumor volume ± standard deviation ($n = 6$). (**E**) Histological and immunohistochemical examination of the resultant tumors. Note that the xenograft tumors exhibited histological and immunohistochemical features similar to those of PDACs. Scale bars represent 50 μm.

telomerase is activated, do not greatly affect the resultant phenotypes of the tumors.

On the other hand, transduction of p53shRNA and KRAS^{G12V} in HPDECs immortalized with CDK4^{R24C}, cyclin D1 and TERT also formed tumors but only after a long latent period. Thus, it is clear that MYC expression facilitated tumor formation. This result is consistent with a previous report that HPV16 E6/E7 and oncogenic KRAS-expressing pancreatic cells showed inconsistent and rather weak tumorigenic potential (21). In the literature, resultant tumorigenic cells have been reported to show activation of AKT and the nuclear factor-κB pathway, which are also observed in PDACs (30,31). Overexpression of NFAT, activation of NOTCH signaling and Wnt/beta-catenin signaling are further features of PDACs (32–34). The available findings point to MYC as a possible convergent downstream target of these different pathways. Indeed, numerous studies have revealed that MYC gene amplification and overexpression contribute to malignant phenotype, and to progression of tumors (35,36).

We closely examined the remaining tissue after tumor regression by DOX administration in order to confirm that no tumor cells remained. Surprisingly, many ductal structures consisting of simple columnar epithelium were apparent. During regression, most tumor cells exhibited features of apoptotic cell death upon DOX administration (Supplementary Figure 3, available at *Carcinogenesis* Online). However, some survived to reform quasinormal ductal structures. Such cells were histologically attached to the basement membrane-like structures. Therefore, it is possible that only cells in a niche which can support survival of normal cells remained. These results might have important implications. Once driver oncogenes could be completely shut-off with specific inhibitors, tumor cells could reverse to normal. Reconstitution of normal tissue architectures of human cells *ex vivo* could be generally obtained by transplantation of the cells in the renal capsule of immunodeficient mice. However, our model system with reversible control of oncogene expression, to our knowledge for the first time, enabled quasinormal human pancreatic duct tissues to be formed subcutaneously in mice.

In recent years, development of drugs targeting RAS and MYC has been reported (37–39). However, normal cells could relapse when administration of the drugs was terminated. This seems to be the situation with many kinase inhibitors, even in the greatest success case of chronic myeloid leukemia treated with imatinib mesylate (Gleevec). To overcome such relapse, considerations of oncogene addiction and synthetic lethality have been emphasized to develop new therapeutic drugs (40). Indeed, the use of experimentally immortalized and transformed mammary epithelial cells has succeeded in identifying selective inhibitors of cancer stem cells using high-throughput screening (41). Since our methodology allows propagation of both normal and cancer-initiating cells under the same conditions *in vitro*, it should provide an ideal set of cells for screening of drugs for this purpose. Also it enabled *de novo* development of PDAC from preformed quasinormal human tissues and might be applicable to carcinogenesis models of any cancers.

Supplementary material

Supplementary Table 1 and Figures 1–5 can be found at http://carcin.oxfordjournals.org/

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molecular pharmaceutics

Brief Article

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Development of a Novel Efficient Method To Construct an Adenovirus Library Displaying Random Peptides on the Fiber Knob

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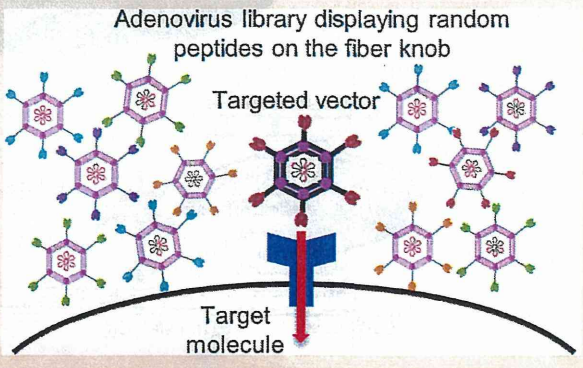
ABSTRACT: Redirection of adenovirus vectors by engineering the capsid-coding region has shown limited success because proper targeting ligands are generally unknown. To overcome this limitation, we constructed an adenovirus library displaying random peptides on the fiber knob, and its screening led to successful selections of several particular targeted vectors. In the previous library construction method, the full length of an adenoviral genome was generated by a Cre-lox mediated *in vitro* recombination between a fiber-modified plasmid library and the enzyme-digested adenoviral DNA/terminal protein complex (DNA-TPC) before transfection to the producer cells. In this system, the procedures were complicated and time-consuming, and approximately 30% of the vectors in the library were defective with no displaying peptide. These may hinder further extensive exploration of cancer-targeting vectors. To resolve these problems, in this study, we developed a novel method with the transfection of a fiber-modified plasmid library and a fiberless adenoviral DNA-TPC in Cre-expressing 293 cells. The use of in-cell Cre recombination and fiberless adenovirus greatly simplified the library-making steps. The fiberless adenovirus was useful in suppressing the expansion of unnecessary adenovirus vectors. In addition, the complexity of the library was more than a 10^4 level in one well in a 6-well dish, which was 10-fold higher than that of the original method. The results demonstrated that this novel method is useful in producing a high quality live adenovirus library, which could facilitate the development of targeted adenovirus vectors for a variety of applications in medicine.



KEYWORDS: *adenovirus, library, Cre recombination, fiberless, targeting vector*

INTRODUCTION

An adenovirus is a potent gene-delivery vehicle and has frequently been used for designing oncolytic viruses.[1−3] However, the lack of specificity on infection due to the widespread distribution of the primary cellular receptor of the adenovirus has prevented the achievement of cancer-specific gene transduction after intravenous administration.[4,5] Also, many cancer cells lack sufficient receptor expression for realizing a therapeutic effect.[4,5] Strategies for redirecting the tropism of the adenovirus vector are therefore being developed to enable efficient target gene delivery to specific cell types.[1,2,6]

Most of the presently used adenovirus vectors are based on serotype 5 (Ad5). Targeting of Ad5 vectors has been achieved by direct genetic modifications of the capsid proteins: targeting ligands can be incorporated into the fiber proteins ablated for native tropism, and these vectors provide an important platform for evaluating the targeting potential of selected peptide ligands.[7−10] The lack of cell-type specific ligands for targeted adenovirus vectors impedes the application of fiber-modified adenovirus vectors for targeted therapies. Although a phage display library has been used to identify targeting peptide motifs, the incorporation of the peptides selected by phage display into the adenoviral capsid is difficult and has not been successful for few cases,[11−14] possibly due to the unwanted conformational change of the virus capsid protein induced by the inserted peptide or the loss of affinity after ligand incorporation.[15,16]

To overcome this limitation, we have developed a system generated by Cre/lox-mediated *in vitro* recombination between an adenoviral fiber-modified plasmid library and a right end-digested adenoviral DNA-terminal protein complex (DNA-TPC), for producing adenoviral libraries displaying a variety of peptides on the fiber knob (Figure 1A).[16,17] We screened the

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