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REVIEW

Pharmaceutical and nutraceutical approaches for preventing liver carcinogenesis: Chemoprevention of hepatocellular carcinoma using acyclic retinoid and branched-chain amino acids

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The poor prognosis for patients with hepatocellular carcinoma (HCC) is associated with its high rate of recurrence in the cirrhotic liver. Therefore, more effective strategies need to be urgently developed for the chemoprevention of this malignancy. The malfunction of retinoid X receptor α, a retinoid receptor, due to phosphorylation by Ras/mitogen-activated protein kinase is closely associated with liver carcinogenesis and may be a promising target for HCC chemoprevention. Acyclic retinoid (ACR), a synthetic retinoid, can prevent HCC development by inhibiting retinoid X receptor α phosphorylation and improve the prognosis for this malignancy. Supplementation with branched-chain amino acids (BCAA), which are used to improve protein malnutrition in patients with liver cirrhosis, can also reduce the risk of HCC in obese cirrhotic patients. In experimental studies, both ACR and BCAA exert suppressive effects on HCC development and the growth of HCC cells. In particular, combined treatment with ACR and BCAA cooperatively inhibits the growth of HCC cells. Furthermore, ACR and BCAA inhibit liver tumorigenesis associated with obesity and diabetes, both of which are critical risk factors for HCC development. These findings suggest that pharmaceutical and nutraceutical approaches using ACR and BCAA may be promising strategies for preventing HCC and improving the prognosis of this malignancy.

Keywords:
Acyclic retinoid / Branched-chain amino acid / Chemoprevention / Hepatocellular carcinoma / Obesity

1 Introduction

Hepatocellular carcinoma (HCC), which usually develops in the livers of patients with chronic hepatitis and liver cirrhosis, is a serious clinical and social issue worldwide. Annually, the number of new cases is approximately 750 000, with an estimated 700 000 patients dying because of the malignancy [1, 2]. Although effective methods of diagnosis and treatment for HCC have been recently developed, improvement in the prognosis for this cancer is limited; overall survival, 10 years after curative treatment, is only 22–35% [3, 4]. The primary reason for the poor prognosis of HCC is its high frequency of recurrence after curative treatment; the recurrence rate, 5 years after definitive therapy in cirrhotic patients, may exceed 70% [5–7]. These facts indicate that curative treatment for HCC is difficult once this malignancy has developed, and therefore, effective strategies for preventing this cancer are urgently required.

In a previous, prospective, randomized trial, we reported that the oral administration of acyclic retinoid (ACR), a novel synthetic retinoid, significantly suppressed the posttherapeutic recurrence of HCC and improved the survival rate of patients [8–10]. Oral supplementation with branched-chain

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Abbreviations: ACR, acyclic retinoid; **BCAA**, branched-chain amino acids; **ERK**, extracellular signal-regulated kinase; **HBV**, hepatitis B virus; **HCC**, hepatocellular carcinoma; **HCV**, hepatitis C virus; **IGF**, insulin-like growth factor; **IGF-1R**, IGF-1 receptor; **MAPK**, mitogen-activated protein kinase; **PEM**, protein energy malnutrition; **PI3K**, phosphoinositide-3-kinase; **RAR**, retinoic acid receptor; **RTK**, receptor tyrosine kinase; **RXR**, retinoid X receptor

amino acids (BCAA), which is widely used in patients with liver cirrhosis to improve protein energy malnutrition (PEM), also reduced the risk of HCC in obese cirrhotic patients [11]. The effects of ACR and BCAA on the chemoprevention of HCC and the inhibition of HCC cell growth have been reported in several experimental studies [12–16]. In particular, recent rodent studies demonstrated that administration of ACR and BCAA suppresses the liver carcinogenesis associated with obesity and diabetes, both of which are critical risk factors for HCC development [17, 18]. The results of these clinical and basic studies strongly suggest that pharmaceutical and nutraceutical approaches, especially using ACR and BCAA, might be effective strategies for preventing liver carcinogenesis. In this article, we provide an overview of the clinical characteristics and molecular pathogenesis of HCC, focusing on the role of retinoid X receptor α (RXRα) phosphorylation in liver carcinogenesis. The detailed effects of ACR and BCAA in the prevention of HCC development are reviewed, based on our clinical and basic research. We also review the possibility of pharmaceutical and nutraceutical approaches for the inhibition of obesity- and diabetes-related liver carcinogenesis through the targeting of the pathophysiological conditions caused by these metabolic abnormalities, concentrating on the effects of ACR and BCAA.

2 Clinical characteristics of HCC

Most cases of HCC, which is the dominant form of primary liver carcinoma, are associated with the chronic inflammation and subsequent cirrhosis of the liver, that is induced by a persistent infection with one of the hepatitis viruses, hepatitis B virus (HBV) or hepatitis C virus (HCV) [19, 20]. After development of virus-induced chronic hepatitis and liver cirrhosis, the entire liver enters a precancerous state, possessing multiple, independent, premalignant, or latent malignant clones. Therefore, the typical clinical pattern of liver carcinogenesis is **ulticentric carcinogenesis, which is also described as **ield cancerization. This carcinogenesis pattern contributes to the high frequency of HCC development in patients with viral liver cirrhosis. Significantly, the annual rate for HCC development is approximately 7% in cirrhotic patients, and even after curative treatment, the annual incidence of recurrence is approximately 20–25% [5–7]. These facts highlight the poor prognosis of viral liver cirrhotic patients and suggest the possibility of improved clinical outcomes if effective strategies are developed for preventing HCC.

One of the most effective approaches for preventing the development of HCC is the eradication of the hepatitis viruses. Several meta-analyses have shown the effectiveness of IFN therapy for preventing HCV-related HCC [21–23], indicating that sustained antiviral response to IFN-based therapy is associated with a reduced risk of developing this malignancy. In addition, IFN treatment might be effective for preventing HCC development in HCV patients, even if sustained antiviral response is not achieved [24]. Antiviral treatments, such as IFN therapy and nucleos(t)ide analog therapy, also prevent the development of HBV-related HCC [25, 26]. These clinical evidences strongly suggest that antiviral treatment is effective for reducing the incidence of HCC development in patients with chronic HBV or HCV infections. In addition, two cohort studies of HCV patients demonstrated that hepatic inflammation alleviation therapy, involving glycyrrhizin injection, suppressed HCC development [27, 28]. These results also indicate that attenuation of chronic inflammation might be effective for inhibiting liver carcinogenesis.

3 Molecular pathogenesis of HCC

HCC is a heterogeneous tumor because it develops in a complex multistep process in which many signaling cascades are altered. That is, the accumulation of genetic alterations is critically involved in hepatocarcinogenesis [29, 30]. Genomic mutations in the *p53* tumor suppressor gene occur in 10–35% of HCC cases [31]. Genomic mutations in the *CTNNB1* gene, which encodes β-catenin, have also identified in approximately 20–40% of liver cancers [31]. Because of these alterations, several signaling pathways related to cell proliferation and survival are activated during liver carcinogenesis. For instance, epithelial growth factor receptor, which is a receptor tyrosine kinase (RTK), is expressed in 68% of HCC cases, and this receptor is associated with the proliferation and clinical stage of this malignancy [32]. Activation of insulin-like growth factor (IGF) 1 receptor (IGF-1R) signaling, which is another RTK, also contributes to the early stages of liver carcinogenesis [30]. The major signaling pathways activated by the RTKs/Ras pathways are the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and the phosphoinositide-3-kinase (PI3K)/Akt pathways, both of which play important roles in the proliferation and survival of cancer cells. These reports, therefore, strongly suggest that targeting specific RTKs and their downstream signaling pathways is a potentially effective strategy for preventing some types of human malignancies, including HCC [33–38].

4 Retinoid abnormalities and HCC

In addition to the pathophysiological mechanisms as mentioned above, recent studies have revealed the magnitude of the abnormalities in the expression and function of retinoids on liver carcinogenesis [12–15, 39]. Retinoids are a group of natural and synthetic molecules that are structurally and/or functionally related to fat-soluble vitamin A. These molecules participate in a broad spectrum of biological activities, including embryogenesis, growth, differentiation, proliferation, apoptosis, and metabolism [40–42]. The fundamental effects of retinoids on cellular activities are largely mediated through the expression of two distinct families of nuclear receptors, the retinoic acid receptors (RARs) and RXRs. The RARs are activated by all-*trans*-retinoic acid and 9-*cis*-retinoic

acid, with similar affinities, whereas RXRs are only activated by 9-*cis*-retinoic acid [40–42]. Both the RARs and RXRs are composed of three subtypes (α, β, and γ), which are characterized by a modular domain structure, and these nuclear receptors are ligand-dependent transcription factors [40–42]. After ligand binding, the RXRs form homodimers and heterodimers with the RARs and interact with the retinoid X response element or the RAR responsive element, which are located in the promoter region of the target genes, thereby modulating gene expression [40–42]. RXRs can also form heterodimers with other nuclear receptors, such as peroxisome proliferator-activated receptor, indicating that RXRs act as common heterodimerization partners for various types of nuclear receptors [41]. Thus, RXRs are considered the master regulators of nuclear receptors because they are involved in the regulation of fundamental cell activities, including normal cell proliferation, metabolism, and death (regulation of apoptosis). In particular, RXRα plays a critical role in the normal control of hepatocyte lifespan and proliferation [43, 44].

These characteristics also suggest that abnormalities in the expression and function of retinoid signaling are closely associated with deviations from normal cell proliferation and death, which are key factors in the development of several types of human cancers, including HCC. For example, retinol, a transport form of retinoid in the plasma, is locally deficient in HCC, but not in the adjacent, normal liver tissue in a rodent model of hepatocarcinogenesis [45]. In a rat model of chemically induced liver carcinogenesis, repression of RXRα occurs even in the early stages of carcinogenesis because its expression is decreased not only in HCC and liver cell adenoma, but also in precancerous HCC lesions [46]. The expression levels of RARβ, which is regarded as a tumor suppressor gene because of its ability to regulate cell growth and apoptosis [47], are markedly decreased in both human [48] and rat HCC [46]. On the other hand, RARγ, which is overexpressed in human HCC tissues and cells, enhances the growth of HCC cells through the activation of the PI3K/Akt signaling pathway [49]. These reports strongly indicate that the restoration of the function and expression of retinoid receptors, via treatment with retinoids, might be effective for the prevention of certain types of human malignancies, including HCC [12–15, 50, 51].

5 RXRα phosphorylation and HCC

We proposed that RXRα phosphorylation and its malfunction is closely associated with liver carcinogenesis [12–15]. RXRα protein, which is anomalously phosphorylated at its serine and threonine residues, prominently accumulates in both surgically resected human HCC tissues and human HCC-derived cell lines [39, 52]. Activation of the RTK/Ras/MAPK signaling frequently occurs in HCC cells [30, 32]. The constitutive phosphorylation of serine-260 in RXRα, a MAPK/ERK consensus site, by this signaling pathway is closely associated with retarded degradation of RXRα, lowered transcriptional activity of this nuclear receptor, and promotion of cancer cell growth [39, 53]. In human HCC cells, phosphorylated RXRα is resistant to proteolytic degradation via the ubiquitination-/proteasome-mediated pathway, facilitating the accumulation of this phosphorylated protein within HCC tissues [54]. Furthermore, phosphorylated RXRα abolishes its ability to form heterodimers with RARβ, and this is implicated in uncontrolled cell growth and retinoid resistance [55]. These findings suggest that the accumulation of phosphorylated RXRα, regarded as the nonfunctional form of RXRα, may interfere with the function of normal (unphosphorylated) RXRα in a dominant-negative manner, thus, playing a critical role in liver carcinogenesis (Fig. 1). On the other hand, the abrogation of RXRα phosphorylation by a MAPK inhibitor or transfection with the nonphosphomimetic mutant RXRα restores the degradation of RXRα in a ligand-dependent manner [39, 53]. Thus, the targeting of RXRα phosphorylation might be a strategy for preventing HCC, and ACR is a promising agent for this purpose, as discussed in Section 6.

6 Mechanisms of ACR in HCC chemoprevention

ACR, also known as NIK-333 and Peretinoin (Kowa Pharmaceutical, Tokyo, Japan), is a synthetic retinoid that was initially developed as an agonist for both RXR and RAR [56, 57]. ACR inhibits growth of human HCC-derived cells by activating the promoter activity of retinoid X response element and RAR responsive element and regulating the expression of retinoid target genes, including *RARβ*, $p21^{CIP1}$, and *cyclin D1*, resulting in the induction of apoptosis and cell cycle arrest in the G_0–G_1 phase [53, 58–63]. These findings indicate that ACR exerts growth inhibitory effects in HCC cells, at least in part, by working as a ligand for retinoid receptors and controlling their target genes, especially *RARβ* and $p21^{CIP1}$. The antitumor effects of ACR are also associated with suppression of telomerase activity, attenuation of oxidative stress, and inhibition of angiogenesis [64–66]. Moreover, the suppressive effects of ACR on liver carcinogenesis have been demonstrated in several animal experiments [17, 45, 67–69].

Furthermore, we have proposed that inhibition of RXRα phosphorylation is a critical mechanism of ACR, allowing it to exert chemopreventive effects in liver carcinogenesis. In human HCC-derived cells, ACR can restore RXRα function by inactivating the Ras/MAPK signaling system and dephosphorylating RXRα, although 9-*cis*-retinoic acid is incapable of suppressing ERK and RXRα phosphorylation [53]. Moreover, recent studies have revealed that ACR suppresses the growth of several types of cancer cells, such as HCC and head and neck squamous cell carcinoma cells, and prevents chemically induced liver carcinogenesis by inhibiting the activation and expression of several types of growth factors and their corresponding RTKs [63, 66, 68–73]. ACR also inhibits Ras activation, and this is associated with prevention of obesity-related liver tumorigenesis in mice and the

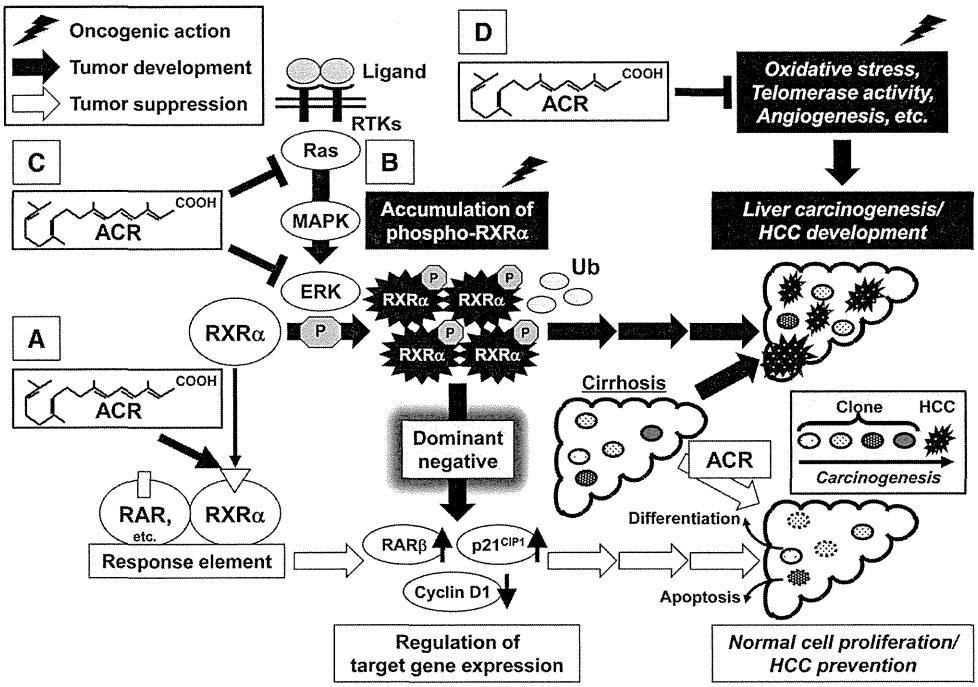

Figure 1. Retinoid refractoriness due to phosphorylation of RXRα, and its restoration by ACR in liver carcinogenesis. When ACR binds to and activates RXRα in normal hepatocytes, the receptor forms homodimers and/or heterodimers with other nuclear receptors, including RARs. This results in the expression of the target genes, such as *RARβ*, *p21^{CIP1}*, and *cyclin D1*, which regulate normal cell proliferation and differentiation and control the induction of apoptosis and cell cycle progression. Therefore, in the cirrhotic liver, ACR can delete and inhibit malignant clones, at least in part, by controlling the expression of these RXRα-target genes (A). In HCC cells, several types of RTKs, such as epidermal growth factor receptor superfamily and IGF-1R and their downstream Ras/MAPK pathway, are highly activated, which results in the phosphorylation of ERK and RXRα and subsequent suppression of dimer formation and transactivation functions of RXRα (refractoriness to retinoid). Furthermore, nonfunctional phosphorylated RXRα, which is sequestered from ubiquitin (Ub)/proteasome-mediated degradation and accumulates in liver cells, interferes with the physiological functions of the remaining nonphosphorylated (i.e., functional) RXRα in a dominant-negative manner, and this is also involved in liver carcinogenesis (B). ACR inhibits phosphorylation of RXRα, restores the function of this receptor, and activates the transcriptional activity of the responsive element associated with this receptor. This is accomplished by inhibiting the Ras/MAPK signaling pathway and the ligand-dependent (growth factor) RTK activities, which contribute to the prevention of liver carcinogenesis and suppression of growth in HCC cells (C). In addition, ACR inhibits growth of HCC cells through the attenuation of oxidative stress, inhibition of telomerase activity, and repression of angiogenesis (D). The pleiotropic effects of ACR to prevent HCC development have also been summarized in recent reviews [12–15].

inhibition of cell growth in human HCC and pancreatic cancer cells [17, 58, 74]. These findings indicate that activation of the RTK/Ras/MAPK signaling pathway, which is involved in HCC development [30, 32], and the subsequent phosphorylation of RXRα are critical targets of ACR for the inhibition of liver carcinogenesis [12–15] (Fig. 1).

7 HCC chemoprevention by ACR: Clinical trial results

Because the results from numerous preclinical experiments indicated that ACR may be an effective agent for HCC chemoprevention, an early-phase, randomized, controlled clinical trial was conducted to determine whether ACR can reduce the incidence and recurrence of second primary HCC in patients who underwent potentially curative treatment for initial HCC [8–10]. In this trial, oral administration of ACR (44 patients, 600 mg/day) for 12 months significantly reduced the incidence of recurrent or new HCC compared to placebo (45 patients) after a median follow-up period of 38 months; 12 patients (27%) in the ACR group developed HCC as compared with 22 patients (49%) in the placebo group ($p = 0.04$) [8]. After a further follow-up period of 62 months, ACR treatment demonstrated improved recurrence-free survival ($p = 0.002$) and overall survival ($p = 0.04$) [9]. The relative risk for the development of secondary HCC and death were 0.31 (95% confidence interval [CI], 0.12–0.78) and 0.33 (95% CI, 0.11–0.79), respectively [8, 9]. Therefore, the estimated 6-year overall survival was 74% in the ACR group and 46% in the placebo group [9].

A multicenter, large-scale ($n = 401$), randomized, placebo-controlled trial also confirmed the effectiveness of ACR in preventing second primary HCCs in HCV-positive patients

who underwent curative treatment for primary or the first recurrence of HCC, with a median follow-up of 2.5 years. In this trial, oral administration of ACR (600 mg/day) had a strong effect on the prevention of a second primary HCC with a hazard ratio of 0.27 (95% CI, 0.07–0.96), 2 years after treatment, and at 3 years, the cumulative recurrence-free survival rates in the ACR-treated group (43.7%) were higher than those in the placebo group (29.3%) [75]. In addition, a subgroup analysis of this study showed that ACR prevented development of a second primary HCC with a hazard ratio of 0.38 (95% CI, 0.20–0.71) in patients who were Child-Pugh A and had small tumors (size, <20 mm) [76]. These results indicated that ACR administration at an early stage of liver cirrhosis contributes to the prevention of HCC. In addition to the effectiveness of ACR for the prevention of HCC development, the results of these clinical trials [8–10,75,76], together with a phase I pharmacokinetics trial [77], have proven the safety of ACR in a clinical setting. Therefore, the findings of these clinical trials [8–10,75–77] strongly suggest that ACR is a novel first-line therapy for reducing the development of a second primary HCC.

8 HCC chemoprevention by ACR: The concept of "clonal deletion" therapy

Two interesting facts were revealed in an early-phase, ACR clinical trial [8–10]. First, the preventive effects of ACR on HCC development lasted up to 50 months after randomization or 38 months after completion of ACR administration, indicating that a 12-month administration of this agent conferred a long-term effect on the prevention of second primary HCCs [10]. Second, ACR administration for 12 months significantly reduced the serum levels of lectin-reactive α-fetoprotein factor 3, which might be produced from latent (i.e., invisible) malignant clones in the remnant liver [78]. These facts suggest the following two possibilities: (i) ACR can delete the α-fetoprotein factor 3 producing premalignant clones from the remnant liver before they expand into clinically detectable HCC and (ii) after the elimination of the malignant clones from the remnant liver by ACR, several years elapse before the clinical appearance of the next HCC clones. The cirrhotic liver is a precancerous field that possesses multiple, independent premalignant, or latent malignant clones. Therefore, before expanding into clinically detectable tumors, a positive approach for the removal and inhibition of such latent malignant clones from the cirrhotic liver should be conducted to prevent HCC development. We consider that implementation of this approach, termed clonal deletion therapy, is a practical approach for preventing HCC, and that ACR is a consistent and reasonable agent for this purpose [12–15] (Fig. 1).

A recent study by Honda et al. [79] reported that an 8-wk administration of ACR significantly elevated the expression levels of many retinoid target genes and tumor suppressor-related genes, but decreased the expression levels of tumor progression-related genes in the liver of HCV-positive patients. This report may also provide evidence that ACR can change the hepatic environment to a non-hypercarcinogenic one.

9 BCAA supplementation and chronic liver disease

BCAA (valine, leucine, and isoleucine) is a widely accepted therapy for improving hepatic insufficiency and its related PEM, which is a common manifestation of patients with liver cirrhosis [80, 81]. PEM affects the outcome of the cirrhotic patients by determining both their quality of life and survival [82, 83]. Cirrhotic patients frequently demonstrate a decreased serum ratio of BCAA to aromatic amino acids, reduced serum albumin levels, and decreased skeletal muscle volume [80, 81]. They have also demonstrated that an increased consumption of foods containing high BCAA content does not affect plasma BCAA levels [84]. On the other hand, nutritional intervention with BCAA has been shown to increase the serum albumin concentration and improve patient quality of life and prognosis by preventing severe complications associated with this disease [85–88]. For instance, in a multicenter, large scale ($n = 646$), randomized, and nutrient intake-controlled trial in Japan, the long-term survival study, oral supplementation with BCAA (12 g/day) for 2 years to patients with decompensated cirrhosis significantly decreased the incidence of events associated with progression to hepatic failure (hazard ratio, 0.67; 95% CI, 0.49–0.93; $p = 0.015$; median observation period, 445 days) [85]. The reports of the trial [85–88], therefore, indicated that BCAA supplementation may serve as a first-line therapy for patients with decompensated cirrhosis.

10 HCC chemoprevention by BCAA supplementation

Several experimental studies have revealed the precise mechanisms of BCAA in the suppression of cancer cell growth and chemoprevention of HCC. Hagiwara et al. [89] reported that BCAA directly suppresses HCC cell proliferation by inducing apoptosis and inhibiting the activation of PI3K/Akt and nuclear factor-κB signaling pathways. BCAA treatment also inhibits the proliferation of human HCC-derived cells by increasing cellular levels of $p21^{CIP1}$ and arresting the cell cycle in the G_0/G_1 phase [90]. Both in vitro and in vivo studies have demonstrated the antiangiogenesis activity of BCAA induced by suppressing the expression of vascular endothelial growth factor in HCC cell lines and in the liver of rats bearing neoplasm [91, 92]. BCAA supplementation also reduces oxidative stress in HCV-positive patients with liver cirrhosis as well as in rats with advanced liver cirrhosis [93, 94]. These reports suggest that BCAA exerts chemopreventive effects against HCC, at least in part, by suppressing angiogenesis and

improving oxidative stress, both of which are critically involved in liver carcinogenesis.

Moreover, recent clinical trials revealed that BCAA supplementation may influence the prevention of HCC development [11, 95–100]. The results of a retrospective analysis showed that BCAA supplementation (12 g/day for >6 months) reduced the incidence of HCC in patients with liver cirrhosis with a hazard ratio of 0.42 (95% CI, 0.22–0.80; $p = 0.009$) [95]. Oral supplementation of BCAA (12 g/day for 6 months) significantly decreased the serum levels of AFP and reduced early recurrence after hepatic resection in patients with HCC [98]. In a subset analysis of the long-term survival study, Muto et al. also showed that long-term oral supplementation with BCAA significantly inhibited the development of HCC in type C cirrhotic patients with BMIs >25 [11]. Moreover, the administration of BCAA granules (12 g/day for 60 months) markedly inhibited the cumulative recurrence of HCC, after curative treatment in patients, with insulin resistance [96]. Therefore, long-term treatment with BCAA is an effective strategy for improving the clinical outcomes in cirrhotic patients by reducing the likelihood of liver failure and in obese and diabetic patients, by suppressing liver carcinogenesis. Pathophysiological conditions involved in the development of obesity-related HCC and in the precise mechanisms of BCAA to inhibit liver carcinogenesis, in particular the mechanisms associated with obesity, are discussed in the following sections.

11 Obesity and HCC

Among patients with liver cirrhosis, the proportion of obese subjects is gradually increasing [101, 102]. This is a serious problem when considering the medical care of chronic liver disease because obesity and its related metabolic abnormalities, especially diabetes mellitus, are major risk factors for the development of HCC [11, 103–106]. Nonalcoholic fatty liver disease, a hepatic manifestation of obesity and metabolic syndrome, is also an important healthcare problem, especially in developed countries, since it can progress to nonalcoholic steatohepatitis), which in turn leads to liver cirrhosis and HCC development [107, 108].

Recent studies have shown several pathophysiological mechanisms linking obesity and liver carcinogenesis, including the emergence of insulin resistance, activation of the IGF/IGF-1R axis, development of a state of chronic inflammation, induction of oxidative stress, and adipokine imbalance [103, 104]. In particular, insulin resistance, which leads to systemic and hepatic inflammation, liver steatosis, and activation of the IGF/IGF-1R axis, is considered to play a critical role in the development of HCC [35, 103, 104, 109]. On the other hand, these reports strongly indicate that targeting such pathophysiological disorders via pharmaceutical and nutraceutical intervention might be an effective strategy to prevent obesity-related liver carcinogenesis [16, 110]. For instance, pitavastatin, a drug widely used for the treatment of hyperlipidemia, and (−)-epigallocatechin-3-gallate, one of the green tea catechins, significantly inhibit the obesity-related liver tumorigenesis by attenuating the chronic inflammation induced by excess fat deposition [111, 112]. Administration of ACR also suppresses diethylnitrosamine-induced liver tumorigenesis in obese and diabetic mice and this is associated with inhibition of Ras activation and phosphorylation of the ERK and RXRα proteins [17]. Increase in insulin sensitivity and the attenuation of systemic and hepatic inflammation by ACR also contribute to this inhibition [17], indicating that ACR might be useful in the chemoprevention of obesity-related HCC (Fig. 2).

12 Preventive mechanisms of BCAA in obesity-related liver carcinogenesis

Recent experimental studies have revealed that BCAA improves insulin resistance and glucose tolerance via the enhancement of glucose metabolism in skeletal muscle, adipose tissue, and the liver [113–118]. Improvements in insulin resistance and glucose tolerance, by oral BCAA supplementation in chronic liver disease patients, have also been reported in several clinical trials [119–121]. In addition, a recent in vitro study showed that BCAA treatment suppresses insulin-induced proliferation of HCC cells by inhibiting the insulin-induced activation of the PI3K/Akt pathway and the subsequent antiapoptotic pathway [89]. We, therefore, consider that improvements in glucose metabolism and insulin resistance might be a critical mechanism in the reduction of the incidence of HCC development in obese cirrhotic patients [11]. This hypothesis was evaluated using an obesity- and diabetes-related liver carcinogenesis mouse model [18]. In the model, BCAA supplementation significantly inhibited diethylnitrosamine-induced liver tumorigenesis in obese and diabetic *db/db* mice by improving liver steatosis and fibrosis, insulin resistance, and hyperleptinemia [18]. Supplementation with BCAA also inhibited the spontaneous development of hepatic premalignant lesions in *db/db* mice via the attenuation of chronic inflammation in both the liver and white adipose tissue [122]. Moreover, BCAA treatment significantly inhibited the proliferation of human HCC-derived cells induced by visfatin, a serum adipokine that is significantly correlated with stage progression and tumor enlargement of HCC [90]. Yoshiji et al. [92] also reported that, in obese and diabetic rats exhibiting insulin resistance, BCAA treatment significantly exerted a chemopreventive effect against HCC through the suppression of hepatic neovascularization. The results of these reports [18, 89, 90, 92, 122] strongly indicate that BCAA inhibits obesity-related liver carcinogenesis by targeting insulin resistance and subsequently by reducing chronic inflammation and adipokine imbalance (Fig. 2). In addition to the liver, supplementation with BCAA suppressed obesity- and diabetes-related carcinogenesis in the colorectum, and this was also associated with the improvement of insulin resistance and inhibition of the activation of

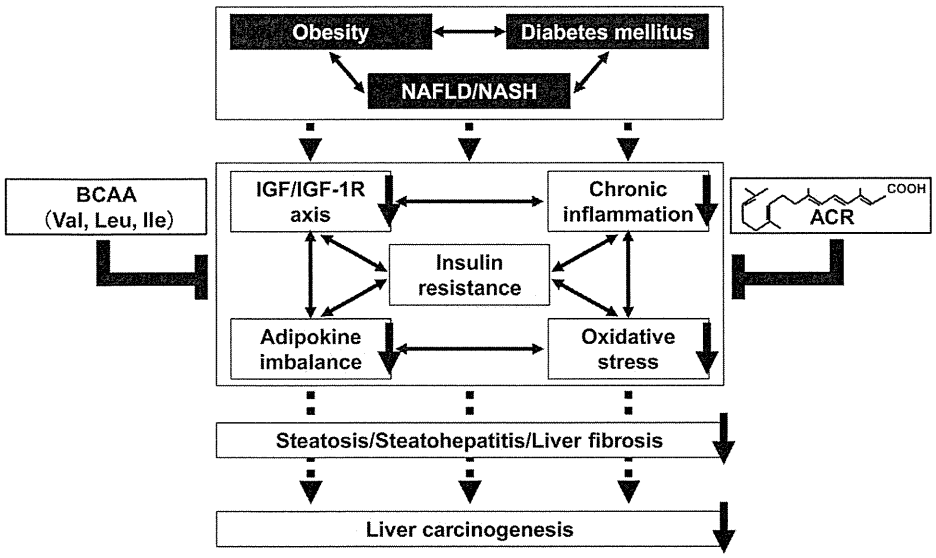

Figure 2. The mechanisms of action of ACR and BCAA in the inhibition of obesity-related liver carcinogenesis. Obesity and diabetes mellitus significantly increase the risk of HCC. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis, which are usually associated with obesity and diabetes, also play critical roles in the development of HCC. Several pathophysiological mechanisms link obesity and liver carcinogenesis, including the emergence of insulin resistance, activation of the IGF/IGF-1R axis, a state of chronic inflammation, induction of oxidative stress, and occurrence of adipokine imbalance. Among them, in particular, insulin resistance plays a key role in obesity-related liver carcinogenesis. Oral supplementation with BCAA significantly reduces the risk of HCC development in obese cirrhotic patients, and this might be associated with decreased insulin resistance and hepatic steatosis, inhibition of the activation of the IGF/IGF-1R axis, and attenuation of oxidative stress and hyperleptinemia. ACR administration also prevents obesity- and diabetes-related liver tumorigenesis in mice by improving hepatic steatosis and insulin resistance, while attenuating chronic inflammation.

the IGF/IGF-IR axis [123]. BCAA, therefore, may be a useful chemoprevention modality for HCC and probably colorectal cancer in obese people.

13 Conclusion

Throughout this review, we have indicated that both ACR and BCAA are promising agents for the prevention of liver carcinogenesis. Therefore, we considered that a combination therapy involving both ACR and BCAA may better inhibit HCC cell growth. Interestingly, a combined ACR and BCAA treatment significantly inhibited the growth of human HCC xenografts in nude mice by inhibiting the phosphorylation of the RXRα, ERK, Akt, and IGF-1R proteins in the xenografts [124]. These results indicated that this combination might be effective for the treatment and probably chemoprevention of HCC. The beneficial effects of the combination approach to chemoprevention, using ACR as a key agent for the prevention and treatment of HCC, have been previously reported [58, 59, 125–127]. A clinical trial also demonstrated that the combination of BCAA and perindopril, an antihypertensive drug, inhibited the cumulative recurrence of HCC after curative therapy and this was associated with improved insulin resistance [128]. Therefore, a combination therapy using ACR and/or BCAA may represent a potential new strategy for chemoprevention of HCC development.

In summary, the poor prognosis of patients with HCC is because of its high incidence and recurrence in cirrhotic livers. Therefore, more effective strategies for the chemoprevention of HCC should be developed to directly improve prognoses for these patients. The results from both experimental and clinical studies strongly suggest that pharmaceutical and nutraceutical approaches, in particular using ACR and BCAA, play a central role in this strategy. These agents may also play a critical role in the prevention of obesity-related liver carcinogenesis, which is a new, serious problem in modern society.

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Cell

Premature Termination of Reprogramming In Vivo Leads to Cancer Development through Altered Epigenetic Regulation

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SUMMARY

Cancer is believed to arise primarily through accumulation of genetic mutations. Although induced pluripotent stem cell (iPSC) generation does not require changes in genomic sequence, iPSCs acquire unlimited growth potential, a characteristic shared with cancer cells. Here, we describe a murine system in which reprogramming factor expression in vivo can be controlled temporally with doxycycline (Dox). Notably, transient expression of reprogramming factors in vivo results in tumor development in various tissues consisting of undifferentiated dysplastic cells exhibiting global changes in DNA methylation patterns. The Dox-withdrawn tumors arising in the kidney share a number of characteristics with Wilms tumor, a common pediatric kidney cancer. We also demonstrate that iPSCs derived from Dox-withdrawn kidney tumor cells give rise to nonneoplastic kidney cells in mice, proving that they have not undergone irreversible genetic transformation. These findings suggest that epigenetic regulation associated with iPSC derivation may drive development of particular types of cancer.

INTRODUCTION

Induced pluripotent stem cells (iPSCs) can be established from differentiated somatic cells by the forced induction of four transcription factors: *Oct3/4*, *Klf4*, *Sox2*, and *c-Myc* (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007; Woltjen et al., 2009). To achieve somatic cell reprogramming, multiple cellular processes act synergistically in a sequential manner (Brambrink et al., 2008; Polo et al., 2012; Samavarchi-Tehrani et al., 2010). Despite extensive studies, the precise mechanism of somatic cell reprogramming still remains unclear (Rais et al., 2013). It is known that non-iPSC-like colonies often appear at the intermediate stage of cellular reprogramming in vitro. In addition, there are several reports describing partial iPSCs that deviate successful reprogramming (Fussner et al., 2011; Mikkelsen et al., 2008; Sridharan et al., 2009). However, the characteristics of such failed reprogramming states are largely unknown, and no study has elucidated the failed reprogramming state from cell types other than fibroblasts.

The process of iPSC derivation shares many characteristics with cancer development. During reprogramming, somatic differentiated cells acquire the properties of self-renewal along with unlimited proliferation and exhibit global alterations of the transcriptional program, which are also critical events during carcinogenesis (Ben-Porath et al., 2008). The metabolic switch to glycolysis that occurs during somatic cell reprogramming is similarly observed in cancer development (Folmes et al., 2011). Such similarities suggest that reprogramming processes and cancer development may be partly promoted by overlapping mechanisms (Hong et al., 2009). Practically, the forced induction of the critical reprogramming factor *Oct3/4* in adult somatic cells results in dysplastic growth in epithelial tissues through the inhibition of cellular differentiation in a manner similar to that in embryonic cells (Hochedlinger et al., 2005). These studies provided a possible link between transcription-factor-mediated reprogramming and cancer development.

To elucidate the involvement of failed reprogramming in cancer development, in the present study, we generated an in vivo reprogramming mouse system using reprogramming factor-inducible alleles and examined the effects of reprogramming factor expression in somatic cells in vivo. We show that failed reprogramming-associated cells behave similarly to cancer cells

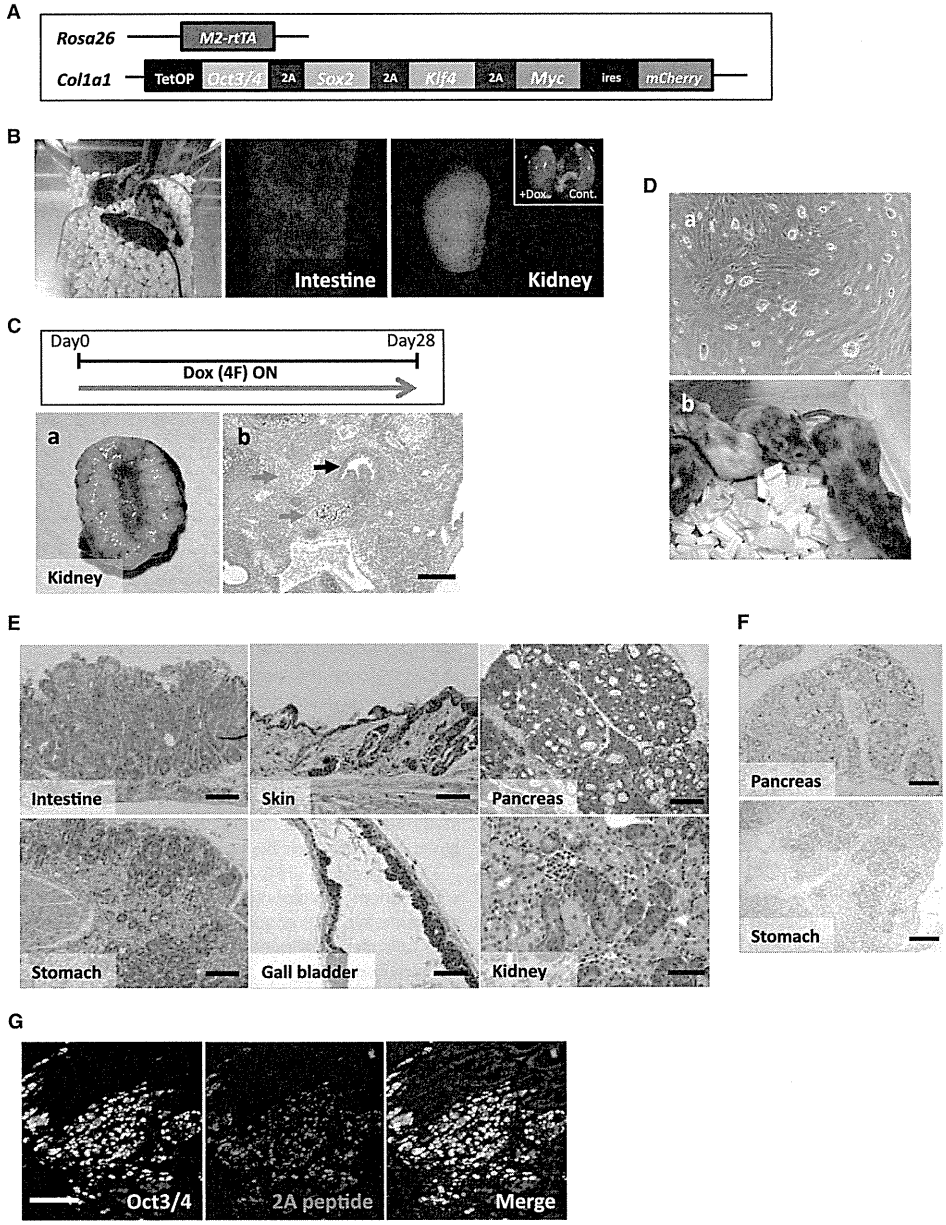

Figure 1. Reprogramming of Somatic Cells In Vivo

(A) Generation of four-factor-inducible ESCs. TetOP, tetracycline-dependent promoter.

(B) Generation of chimeric mice using OSKM-inducible ESCs. mCherry signals could be detected in various organs after Dox treatment for 3 days.

(C) Treatment of chimeric mice with Dox for 28 days resulted in the development of multiple tumors containing pluripotent stem cells. (a) A representative macroscopic image of the cut surface of the kidney tumor. (b) A histological section of the kidney tumor showing the differentiation of tumor cells into three germ layers, indicating teratoma formation. The blue, red, and black arrows represent neuronal, cartilage, and glandular epithelial components, respectively. Scale bar, 200 μm.

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and cause neoplasia resembling Wilms tumor, a childhood blastoma in the kidney. Moreover, we demonstrate that altered epigenetic regulations cause the abnormal growth of such failed reprogramming-associated cancer cells.

RESULTS

In Vivo Reprogrammable Mouse

To establish the reprogrammable mouse system, we generated embryonic stem cells (ESCs) in which reprogramming factors can be induced under the control of doxycycline (Dox) (Figure 1A) (Carey et al., 2010; Stadtfeld et al., 2010b). We used KH2 ESCs with the optimized reverse tetracycline-dependent transactivator at the *ROSA 26* locus (Beard et al., 2006). A polycistronic cassette encoding four reprogramming factors (*Oct3/4*, *Sox2*, *Klf4*, and c-*Myc*) (Carey et al., 2010), followed by *ires-mCherry*, was targeted into the *Col1a1* gene locus under the tetracycline-dependent promoter of KH2 ESCs (Figure 1A).

Next, we generated chimeric mice via blastocyst injection of four-factor (4F)-inducible ESCs. To confirm inducible expression of the reprogramming factors and mCherry in vivo, Dox-containing water was provided to chimeric mice starting at 4 weeks of age. On day 3 of Dox treatment, we could detect the mCherry signal in various organs, including stomach, intestine, liver, pancreas, kidney, gallbladder, and skin (Figure 1B). We also confirmed the expression of reprogramming factors in germline-transmitted mouse tissues by quantitative RT-PCR (qRT-PCR) (Figure S1A available online).

Mouse embryonic fibroblasts (MEFs) containing these reprogramming factor-inducible alleles could give rise to iPSCs after Dox treatment in vitro (Figure S1B). We next asked whether responding somatic cells could be reprogrammed in vivo. The chimeric and germline-transmitted mice given Dox-containing water (2 mg/ml) from 4 weeks of age became morbid within 7–10 days and a few days, respectively. A small proportion of chimeric mice could be treated with Dox for 4 weeks, presumably because of a lower contribution of ESCs in responding tissues. Notably, mice treated with Dox for 4 weeks developed multiple tumors in several organs, such as the kidney and pancreas (Figure 1Ca), whereas tumor formation was never observed in nontreated mice (n = 7, 7 months of age). Histological analysis revealed that these tumors differentiated into three different germ layers, indicating that they are teratomas (Figure 1Cb). When teratoma cells were cultured ex vivo in the absence of Dox (no additional 4F expressions), iPSC-like cells were established (Figure 1Da). Importantly, the teratoma-derived iPSC-like cells contributed to adult chimeric mice when they were injected into blastocysts (Figure 1Db). Therefore, we conclude that somatic cells can be reprogrammed in vivo to pluripotency in our reprogrammable mouse system.

Forced Expression of Reprogramming Factors In Vivo Leads to Rapid Expansion of Dysplastic Cells

We next examined the early changes after expression of reprogramming factors in somatic cells in vivo. After treatment of 4-week-old mice with Dox for 3–9 days, all mice developed dysplastic lesions in epithelial tissues of various organs (Figure 1E), although there were variations in severity of the phenotype among chimeras. Dysplastic cells proliferated actively, as revealed by Ki67 staining (Figure 1F). Abnormal proliferation of somatic cells was observed as early as 3 days after Dox treatment (Figure S1C), and by day 7, such dysplastic cell growth was detected even for pancreatic and kidney cells, which typically do not divide actively under physiological conditions (Figures 1E and 1F). Immunofluorescent analysis of Oct3/4 and the 2A peptide (forming transgene connections) demonstrated that the dysplastic cells expressed reprogramming factors (Figure 1G). Collectively, the forced expression of reprogramming factors caused dysplastic cell expansion of epithelial tissues in vivo.

The Fate of Early Dysplastic Cells after Withdrawal of Dox

To examine whether subsequent expansion of such dysplastic cells depends on the continuous expression of reprogramming factors, we withdrew Dox for 7 days after an initial 4- to 7-day treatment (Figure 2A). Although Dox treatment for 4–7 days caused active cell proliferation in a variety of tissues of all mice, we did not observe any dysplastic cells in some mice after withdrawal of Dox (Figure 2A; Table 1). Of particular note, mice treated with Dox for periods less than 5 days before withdrawal often revealed a lack of dysplastic cells (Table 1). These data suggest that early dysplastic cell growth requires continuous expression of reprogramming factors. We next investigated the fate of eliminated dysplastic proliferating cells after the withdrawal of Dox. Bromodeoxyuridine (BrdU) was injected into mice during Dox treatment to label proliferating cells caused by reprogramming factor expression during the first 7 days (Hochedlinger et al., 2005), and then mice were sacrificed after the withdrawal of Dox for 7 days, on day14. Notably, BrdU-labeled cells were often observed in normal-looking pancreatic and kidney tissues at day14 (Figure 2B). Furthermore, BrdU-labeled cells in the pancreatic islets also expressed insulin (Figure 2B). This suggests that the expanded cells caused by the transient expression of reprogramming factors were, at least in part, integrated into normal-looking tissues after Dox withdrawal.

(D) Teratomas contain pluripotent stem cells. (a) Ex vivo teratoma culture gave rise to iPSC-like colonies without Dox exposure. (b) Teratoma-derived iPSCs contributed to adult chimeric mice.

(E) Dysplastic cell expansion by the forced expression of reprogramming factors in vivo. The histology of various organs of mice treated with Dox for 3 to 9 days. Scale bars, 200 μm (intestine, skin, pancreas, stomach, and gall bladder) and 100 μm (kidney).

(F) Ki67 immunostaining revealed active proliferation of the dysplastic cells in the pancreas and stomach. Scale bars, 200 μm.

(G) Immunofluorescent staining for Oct3/4 and 2A peptide in the intestine of an OSKM chimeric mouse treated with Dox for 7 days. The 2A antibody used here recognizes both Oct3/4-P2A and Sox2-T2A. Dysplastic cells showed positive staining for both Oct3/4 and 2A. Scale bar, 50 μm.

See also Figure S1.

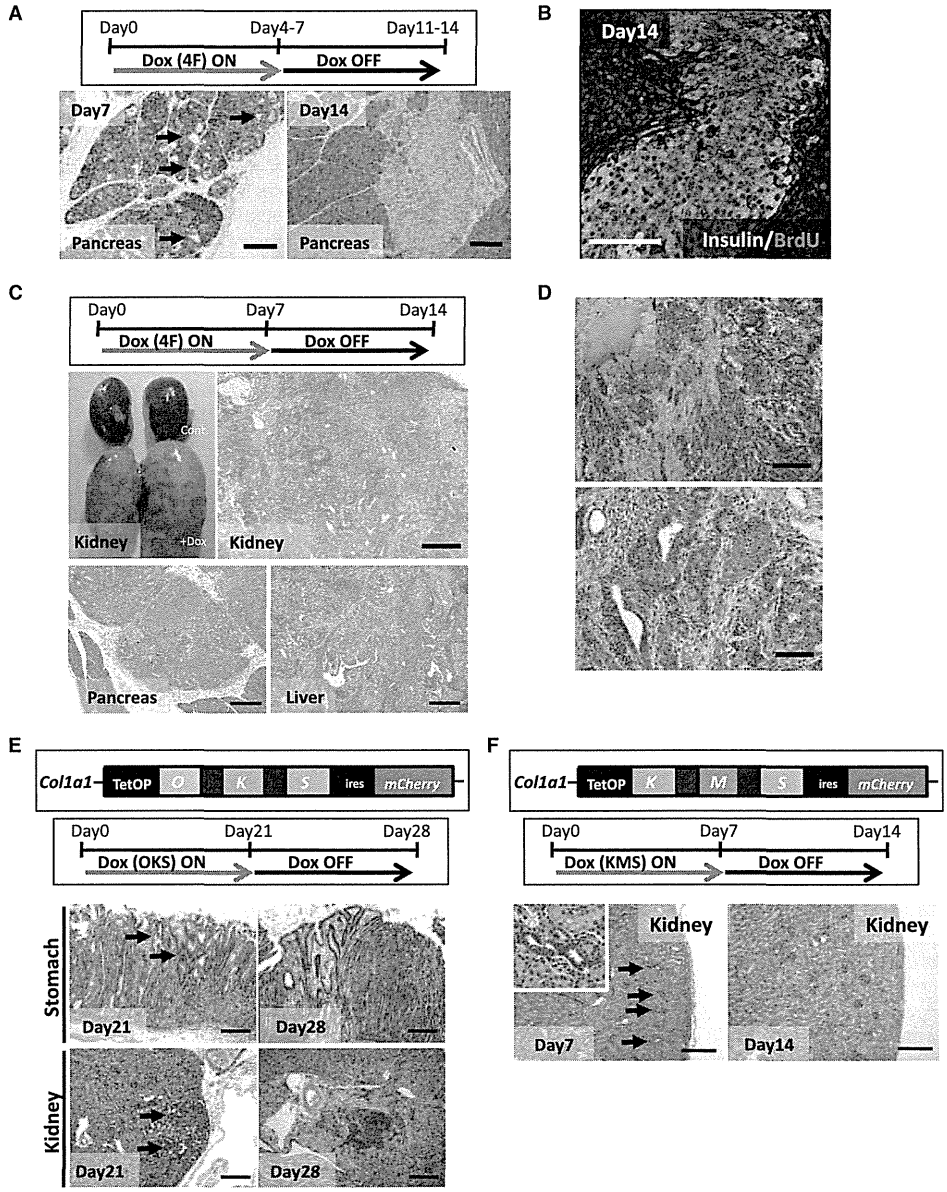

Figure 2. Transient Expression of Reprogramming Factors Causes Neoplasia

(A) A schematic drawing of the experiment and histological sections of the pancreas taken on days 7 and 14. Dysplastic cell growth was induced by treatment with Dox for 7 days (arrows on day 7). The pancreatic section taken on day 14 revealed normal histology. Scale bars, 200 μm.

(B) Double immunofluorescence for insulin and BrdU in the pancreas on day 14. For the pulse and chase experiment, BrdU was injected intraperitoneally every day during Dox administration starting on day 2 (days 2–7), followed by withdrawal of Dox for 7 days. BrdU-positive cells were frequently observed in normal-looking pancreatic islet cells, which also expressed insulin. Scale bar, 100 μm.

(C) Treatment of OSKM chimeric mice with Dox for 7 days, followed by the withdrawal of Dox for another 7 days. The macroscopic image shows the development of bilateral kidney tumors on day 14. Representative histological images are shown for Dox-withdrawn tumors in the kidney, pancreas, and liver. Scale bars, 200 μm.

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Table1. Transient Expression of Reprogramming Factors Causes Tumor Development

		Kidney		Pancreas		Liver	
Dox Treatment	n	No Phenotype	Dysplastic Growth	No Phenotype	Dysplastic Growth	No Phenotype	Dysplastic Growth
4 days ON→OFF	4	2	2	4	0	3	1
5 days ON→OFF	2	1	1	2	0	2	0
6 days ON→OFF	5	1	4	2	3	3	2
7 days ON→OFF	33	7	26	22	11	25	8

Prolonged Expression of Reprogramming Factors Leads to Transgene-Independent Tumor Formation in Somatic Cells

In contrast to the reversion of early dysplastic proliferating cells into normal-looking cells, mice that had been given Dox for 7 days often went on to develop tumors in multiple responding organs even after Dox withdrawal (Figure 2C; Table 1). The developed tumors consisted of histologically undifferentiated dysplastic cells, which were distinct from teratoma cells (Figures 2C and S2A). The dysplastic cells invaded the surrounding tissues, which is one of the hallmarks of cancer cell growth (Figure S2A). Dox-withdrawn tumor cells were negative for 2A staining, affirming that they grew independent of transgene expression (Figure S2B). Dox-withdrawn kidney tumors were similarly observed in elderly mice given Dox starting at 14 weeks of age (13 out of 19 mice). When Dox-withdrawn kidney tumor cells were transplanted into the subcutaneous tissues of immunocompromised mice, they formed secondary tumors within 3 weeks without Dox administration (Figures 2D and S2C), reflecting the neoplastic potential of Dox-withdrawn tumor cells.

Reprogramming factors in our transgenic system include *c-Myc*, a well-known oncogene. To investigate the contribution of *c-Myc* on the development of Dox-withdrawn tumors, we generated three-factor-inducible chimeric mice, which express *Oct3/4*, *Sox2*, and *Klf4* (OKS), but not *c-Myc*, by the targeted insertion of transgenes into the identical locus as 4F (OSKM)-inducible mice (Figure 2E). Similar to 4F-induced mice, OKS induction in vivo caused dysplastic cell growth in various organs yet required longer periods of treatment (Figure 2E). After 3 weeks of induction of OKS followed by withdrawal for 7 days, these mice developed the Dox-withdrawn tumors consisting of undifferentiated dysplastic cells in multiple organs (4 out of 8 mice; Figure 2E). Therefore, transgenic *c-Myc* is dispensable for the development of Dox-withdrawn tumors.

Oct3/4 plays a critical role in cellular reprogramming, and expression of three factors (*Klf4*, *c-Myc*, and *Sox2*) in the absence of *Oct3/4* is not sufficient for iPSC generation (Takahashi and Yamanaka, 2006). To further demonstrate a link between cellular reprogramming and Dox-withdrawn tumor development, we generated chimeric mice in which *Klf4*, c-*Myc*, and *Sox2* (KMS), but not *Oct3/4*, can be induced upon Dox treatment (Figure 2F). Following Dox treatment for 7 days, we observed dysplastic cell growth in the kidney of KMS-inducible mice (three out of six mice; Figure 2F). However, in sharp contrast to OSKM/OKS-induced mice, the withdrawal of Dox eliminated the dysplastic cells in the kidney of KMS-induced mice (n = 17; Figure 2F). A previous study demonstrated that ectopic expression of *Oct3/4* alone can induce dysplastic growth whereas the transgene withdrawal leads to complete reversion of such dysplasia (Hochedlinger et al., 2005). Consistent with the previous observation, the *Oct3/4*-single induction under the same experimental condition failed to form Dox-withdrawn tumors (n = 18; Figure S2D). Taken together, we conclude that reprogramming pressure toward pluripotency driven by the combination of reprogramming factors is associated with the development of Dox-withdrawn tumors.

Loss of Cell Identity and Gain of ESC-Related Gene Expression in Dox-Withdrawn Tumors

To characterize Dox-withdrawn tumor cells, we examined gene expression in kidney tumors that arose in OSKM-inducible mice treated with the 7+/7− Dox regimen. In the KH2 system, transgene expression in the kidney is induced exclusively in the tubule cells (Beard et al., 2006). We observed decreased expression of kidney tubule cell-specific genes in Dox-withdrawn kidney tumors, indicating loss of kidney cell identity (Figure 3A). A previous study dissected the gene expression signature of ESCs into three functional modules: core pluripotency factors, Polycomb complex factors, and Myc-related factors (Kim et al., 2010). Notably, microarray analysis revealed that the ESC-Core module is similarly activated in Dox-withdrawn kidney tumors and ESCs (Figure 3B) (Ohta et al., 2013). We also found that the Myc module displays similar activation between Dox-withdrawn tumors and ESCs (Figure S3A). The activation of ESC-Core and ESC-Myc modules was similarly confirmed in transplanted secondary tumors (Figure S3B).

(D) Minced Dox-withdrawn tumor cells were injected in the subcutaneous tissues of immunocompromised mice. A histological section of one of the tumors phenocopied the original Dox-withdrawn tumor. Scale bars, 200 μm (upper panel) and 100 μm (lower panel).

(E) A schematic drawing of the OKS transgene at the *Col1a1* locus. A histological section of the kidney on days 21 and 28. The expansion of dysplastic cells was observed in the stomach and kidneys on day 21 (arrows). The dysplastic cell growth could be detected even after the withdrawal of Dox in OKS-induced mice (day 28). Scale bars, 200 μm.

(F) A schematic drawing of the KMS transgene. A histological section of a kidney after the treatment with Dox for 7 days (day 7) and the withdrawal of Dox for another 7 days (day 14). KMS induction leads to dysplastic growth in the kidney tubule cells (arrows for day 7). The inset shows a higher-magnification image. No dysplastic cells were detectable in the kidneys of KMS-induced mice after the withdrawal of Dox (day 14). Scale bars, 200 μm.

See also Figure S2.

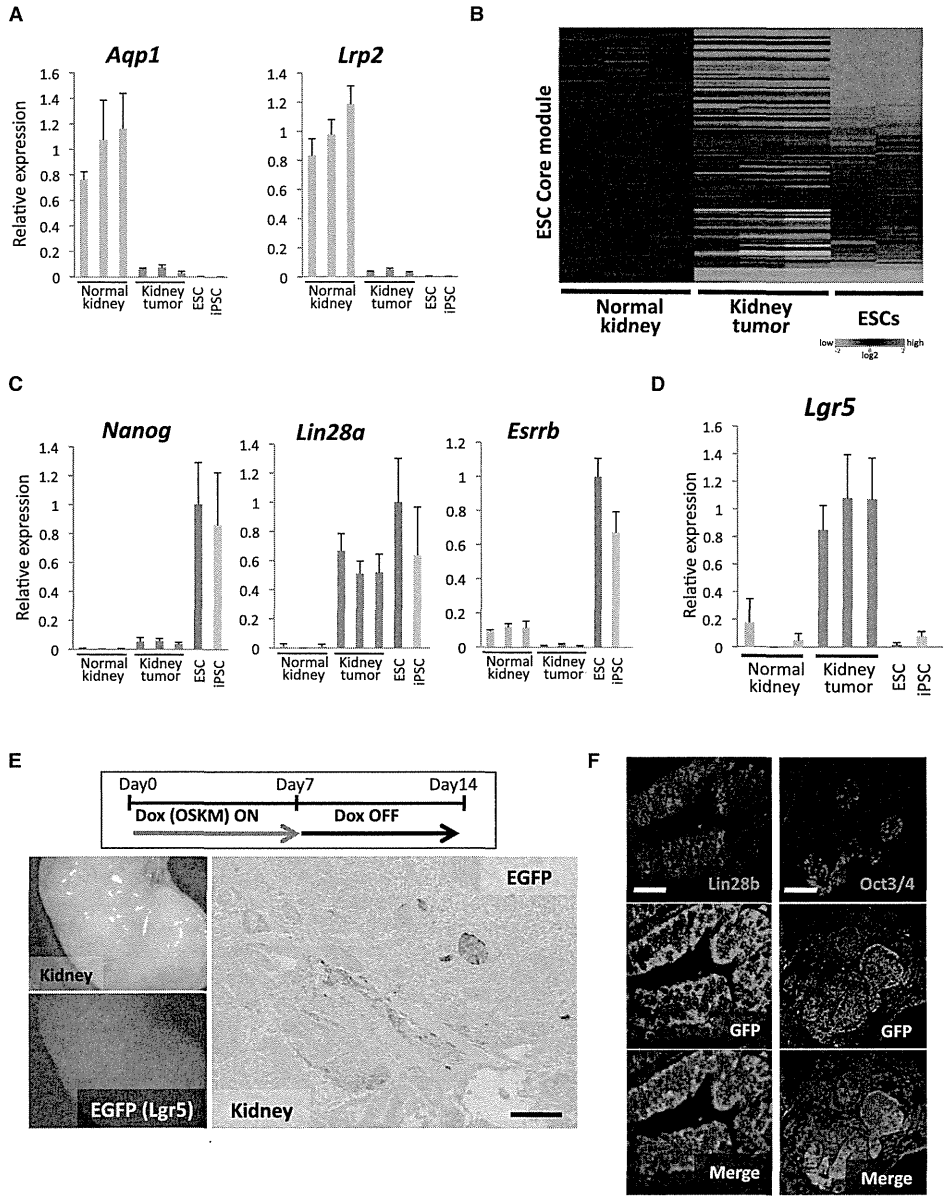

Figure 3. Loss of Cell Identity and Gain of ESC-Related Gene Expression in the Dox-Withdrawn Tumors

(A) The results of the qRT-PCR analyses of *Aqp1* and *Lrp2*. The expression levels of *Aqp1* and *Lrp2* were significantly downregulated in the Dox-withdrawn kidney tumors. Data are presented as mean ± SD. The mean level of normal kidney samples was set to 1.

(B) The microarray analyses revealed the activation of the ESC Core module in Dox-withdrawn kidney tumors.

(C) The results of the qRT-PCR analyses of pluripotency-related genes. Data are presented as mean ± SD. The transcript level in ESCs was set to 1.

(D) *Lgr5* as a candidate marker of Dox-withdrawn kidney tumor cells. *Lgr5* was specifically expressed in Dox-withdrawn kidney tumors. Data are presented as mean ± SD. The mean level of kidney tumors was set to 1.

(E) A schematic drawing of the experimental protocol using chimeric mice with both reprogrammable alleles and the *Lgr5-EGFP* allele. Macroscopic images of the Dox-withdrawn kidney tumor with the *Lgr5-EGFP* allele showing scattered EGFP signals in the kidney tumor. GFP immunostaining of kidney tumor sections revealed that the GFP signals are detectable specifically in tumor cells. Scale bar, 100 μm.

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