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ORIGINAL PAPER

Role of the BLT2, a leukotriene B_4 receptor, in Ras transformation

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Oncogenic Ras is known to drive both the Rac and Raf-MAP-kinase pathways, which act in concert to cause cell transformation. Unlike the Raf-MAP-kinase cascade, however, the downstream elements of Rac pathway are not fully understood. Previously, we showed that cytosolic phospholipase A_2 ($cPLA_2$) and subsequent metabolism of arachidonic acid act downstream of Rac to mediate the transformation signaling induced by Ha-Ras^{V12}. In the present study, we observed that leukotriene B_4 (LTB_4) and its synthetic enzymes as well as BLT2, the low-affinity LTB_4 receptor, are all elevated in Ha-Ras^{V12}-transformed cells. In addition, the malignant phenotypes of Ras-transformed cells were markedly inhibited by BLT2 blockade, as was their tumorigenicity *in vivo*. Finally, *in situ* hybridization analysis revealed that expression of BLT2 is significantly upregulated in a variety of human cancers. Taken together, our results suggest that an LTB_4-BLT2-linked cascade plays a crucial mediatory role in the cell transformation induced by oncogenic Ha-Ras^{V12}, possibly acting downstream of Rac-$cPLA_2$.
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Introduction

Ras proteins have been implicated as key intermediates that mediate the signal from upstream activators, such as growth factor receptors and nonreceptor tyrosine kinases, to various downstream effectors (Barbacid, 1987; Leevers, 1996). The best characterized Ras-activated pathway involves a Raf-mitogen-activated protein kinase (MAPK) cascade that includes Raf1, MAPK kinase and extracellular signal-regulated kinases 1 and 2 (Gauthier-Rouviere *et al*., 1990; Leevers and Marshall, 1992; Marais *et al*., 1995). Activation of this cascade in turn leads to activation of transcription factors such as p62^{TCF}/Elk-1 (Gille *et al*., 1992; Marais *et al*., 1995; Latinkic *et al*., 1996), which then interacts with serum response factor (SRF) at the serum response element (SRE) of the *c-fos* promoter, inducing transcription of *c-fos* in response to MAPK activation.

Ras is believed to also act via a Rac-linked pathway (Qiu *et al*., 1995; Joneson *et al*., 1996). Rac is a member of Rho family GTPases once thought to be involved primarily in organizing the actin cytoskeleton (Hall, 1994). Over the past several years, however, it has become evident that Rac also carries out critical functions in the control of cell proliferation and *c-fos* SRE activation (Ridley *et al*., 1992; Gille *et al*., 1995; Hill *et al*., 1995; Marais *et al*., 1995; Latinkic *et al*., 1996). Consistent with the role of Rac as a downstream Ras effector, cooperation between Rac and Raf-MAPK cascades has been shown to synergistically facilitate cell transformation (Joneson *et al*., 1996). In addition, Rat-1 fibroblasts expressing Rac^{V12}, a constitutively active Rac1 mutant, display many features characteristic of malignant transformation (Qiu *et al*., 1995), again supporting the role of Rac as a downstream mediator of Ras signaling in the pathway leading to cell transformation.

Unlike the Raf-MAPK cascade, the elements downstream of Rac leading to cell transformation are not yet fully understood. It is known, however, that when activated Rac in turn activates cytosolic phospholipase A_2 ($cPLA_2$), which catalyses the hydrolysis of membrane phospholipids leading to the release of the mediator arachidonic acid (AA) (Peppelenbosch *et al*., 1995; Kim and Kim, 1997), and that $cPLA_2$ is also necessary for the Rac signaling leading to SRE activation and transformation (Kim and Kim, 1997; Yoo *et al*., 2001). For instance, inhibition of $cPLA_2$ using mepacrine, a potent PLA_2 inhibitor, or cotransfection with antisense $cPLA_2$ oligonucleotides dramatically represses Rac^{V12}-induced SRE activation (Kim and Kim, 1997). In addition, pretreating cells with mepacrine inhibits such Ha-Ras^{V12}-mediated transformed phenotypes as uncontrolled growth, morphological changes and anchorage-independent growth (Yoo *et al*., 2001). Based on these results, it was suggested that $cPLA_2$ acts downstream of Rac to mediate the signaling leading to malignant transformation induced by Ha-Ras^{V12} (Yoo *et al*., 2001).

AA, a principal product of Rac-activated $cPLA_2$, is metabolized to eicosanoids such as leukotrienes (LTs) via 5-lipoxygenase (5-LO) and 5-lipoxygenase activating protein (FLAP) (Serhan *et al*., 1996; Yokomizo *et al*., 2001a, b; Izumi *et al*., 2002) or to prostanoids via cyclooxygenases (COXs). Notably, LTs including leukotriene B_4 (LTB_4) were speculated to be situated

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downstream of the Rac-cPLA$_2$ cascade, possibly mediating SRE activation or EGF-induced mitogenesis (Peppelenbosch *et al*., 1995; Kim and Kim, 1997; Kim *et al*., 1998). For example, we previously observed that pretreatment with NDGA, a LO inhibitor, but not indomethacin, a COX inhibitor, suppresses SRE activation in response to Rac^{V12} transfection or exogenous AA (Kim and Kim, 1997). Similarly, Rac-mediated AA release and subsequent AA metabolism by 5-LO were shown to be the major pathways by which EGF induces mitogenesis or cytoskeletal rearrangements (Peppelenbosch *et al*., 1995; Kim *et al*., 1998). In addition, inhibition of 5-LO, but not COX, reportedly suppresses growth of Rat-HO6 cells, a transformed Rat2 cell line stably expressing Ha-Ras^{V12} (Yoo *et al*., 2001), suggesting the potential role for LTs as downstream mediators of Ha-Ras^{V12} signaling to transformation. In support of this idea, it has been previously shown that blockade of the 5-LO–LTs pathway inhibits carbamate-induced lung tumors in mice (Gunning *et al*., 2002); that 5-LO and LTB$_4$ receptors are expressed in human pancreatic cancers, but not in normal pancreatic duct tissue (Hennig *et al*., 2002); and that the LTB$_4$ receptor antagonist LY293111 inhibits cell proliferation and induces apoptosis in human pancreatic cancer cells (Tong *et al*., 2002).

With that as background, we investigated the potential role of LTB$_4$ and its receptors in Ha-Ras^{V12}-induced signaling leading to malignant transformation. Our results clearly indicate that levels of LTB$_4$ and its synthetic enzymes as well as BLT2, the low-affinity LTB$_4$ receptor, are highly elevated in Ras-transformed cells. In addition, inhibition of BLT2 using specific BLT2 antagonists could suppress all the features of Ras transformation including tumor formation in athymic mice, suggesting a critical mediatory role of BLT2 in the Ha-Ras^{V12}-induced cell transformation. Consistent with this idea, BLT2 overexpression itself was shown to elicit partial transformed phenotypes. Additionally, *in situ* hybridization analysis revealed that expression of BLT2 is significantly upregulated in a variety of human cancers, suggesting physiological roles of LTB$_4$ and its receptor, BLT2, in the molecular mechanism leading to tumorigenicity *in vivo*.

Results

Enhanced LTB$_4$ production in Ha-Ras^{V12} transformed cells

As a first step in evaluating the possible involvement of LTB$_4$ in Ha-Ras^{V12}-induced cell transformation, we measured LTB$_4$ production from normal Rat2 and Rat2-HO6, a transformed Rat2 cell line expressing Ha-Ras^{V12} (Kumar *et al*., 1992), and found that the latter produced approximately five times more LTB$_4$ than the former (Figure 1). Moreover, when we then examined expression of cPLA$_2$, 5-LO and FLAP, the key proteins involved in LTB$_4$ synthesis, we found that expression of all the three was elevated in Rat2-HO6 cells (Figure 2a).

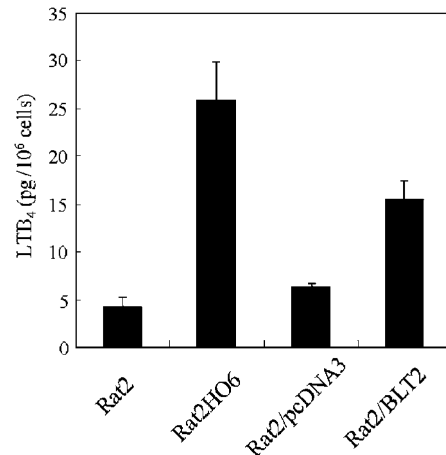

Figure 1 Elevated LTB$_4$ production in Ha-Ras^{V12}-transformed cells. Rat2, Rat2-HO6, Rat2/pcDNA3 and Rat2/BLT2 cells were seeded onto 60 mm dishes and incubated in DMEM containing 0.5% FBS for 24 h. Conditioned media were then harvested and assayed for LTB$_4$ using a specific ELISA, as described in Materials and methods. Data are expressed as mean percentages $\pm$ s.d. of control from three independent experiments

Apparently, levels of both LTB$_4$ and its synthetic enzymes are significantly increased in Ras-transformed cells. As many eicosanoids, including LTB$_4$, often act via specific receptors to exert an autocrine/paracrine effect, the finding of increased LTB$_4$ production in Ras-transformed cells prompted us to determine whether levels of LTB$_4$ receptors are also elevated. To address that question, we used RT–PCR to evaluate the mRNA expression of the LTB$_4$ receptors, BLT1 and BLT2, in Rat2 and Rat2-HO6 cells. BLT1 mRNA was not detected in both cells (data not shown), which is consistent with earlier reports indicating BLT1 to be expressed exclusively in inflammatory leukocytes (Yokomizo *et al*., 1997). On the other hand, Rat2 fibroblasts expressed moderate levels of BLT2 mRNA, and much higher levels were expressed by Rat2-HO6 cells (Figure 2b), suggesting that LTB$_4$ might act in an autocrine/paracrine fashion via its low-affinity receptor (BLT2) to stimulate Ras-mediated transformation. It was therefore noteworthy that Rat2 cells stably expressing BLT2 (Rat2/BLT2) showed produced ~2.5 times more LTB$_4$ than control cells transfected with empty vector (Rat2/pcDNA) (Figure 1), suggesting a potential cross-amplifying regulation between BLT2 and its agonist LTB$_4$. Additionally, we have performed a transient transfection experiment using Ha-Ras^{V12} expression plasmid and, similar to that by the stable Rat2-HO6 cells, Ha-Ras^{V12} transient transfection caused a substantial increase in the expression of BLT2 (Figure 2c).

BLT2 is required for Ras transformation

To further examine the involvement of BLT2 in Ha-Ras^{V12}-induced transformation, we investigated whether

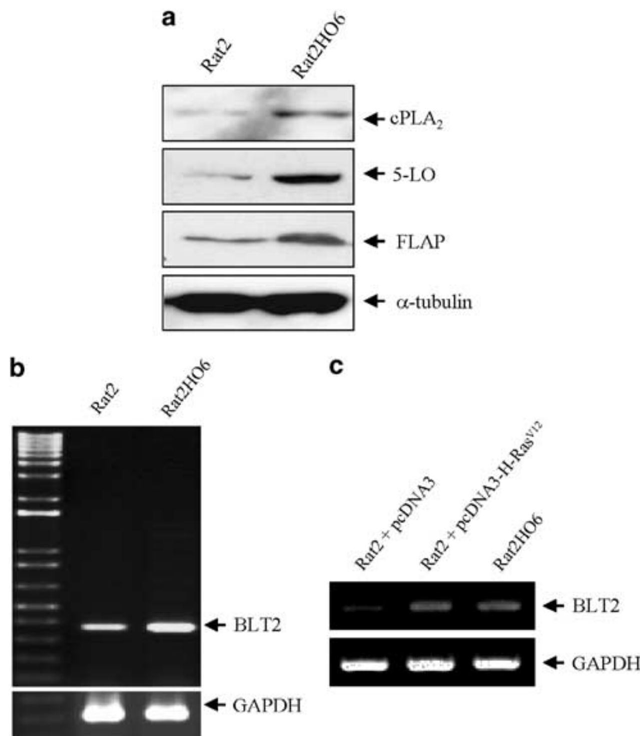

Figure 2 Induced expression of $cPLA_2$, 5-LO, FLAP and BLT2 in Ha-Ras^{V12}-transformed cells. (**a**) Western blot showing expression of $cPLA_2$, 5-LO and FLAP proteins in Rat2 and Rat2-HO6 cells. Cells were plated on 60 mm culture plates and harvested after incubating 24 h. (**b**, **c**) RT–PCR analysis showing relative levels of BLT2 mRNA expression in Rat2 and Rat2-HO6 cells (**b**) and Rat2 cells transfected with 3 μg pcDNA3 or pcDNA3-H-Ras^{V12} (Yoo *et al*., 2001) (**c**). Transient transfection (5×10^5 cells in 100-mm dishes) was carried out as described previously (Yoo *et al*., 2001) and then incubated in fresh DMEM supplemented with 0.5% FBS for an additional 24 h, after which transfected cells as well as control Rat2-HO6 cells were harvested for BLT2 mRNA transcript analysis. Total RNA was reverse transcribed and PCR reactions were performed with rat BLT2-specific primers as described in Materials and methods. Levels of GAPDH were shown as a control. The results shown are representative of three independent experiments with similar results

the BLT antagonist leukotriene B_4-3-aminopropylamide (LTB_4-APA) (Goldman *et al*., 1991; Woo *et al*., 2003) elicits any suppressive effect on transformed phenotypes in Rat2-HO6 cells. We found that the growth rate of Rat2-HO6 cells was higher than that of normal Rat2 cells, and that treatment with LTB_4-APA (500 nM) reduced the growth rate of the former to the same levels seen in the latter, without affecting the growth rate of Rat2 cells themselves (Figure 3a). We then carried out soft agar assays to examine the involvement of LTB_4 in anchorage-independent growth of Rat2-HO6 cells. As expected, normal Rat2 cells were unable to grow on soft agar (data not shown). On the other hand, Rat2-HO6 cells formed numerous colonies on the gel-like material, and their growth was dose-dependently suppressed by LTB_4-APA (Figure 3b). Finally, we found that LTB_4-APA elicited a change in Rat2-HO6 cell morphology, reverting the cells to assume a more flattened shape, similar to that of normal Rat-2 cells (Figure 3c). Together, these results suggest that BLT2 is required for Ras-induced transformation.

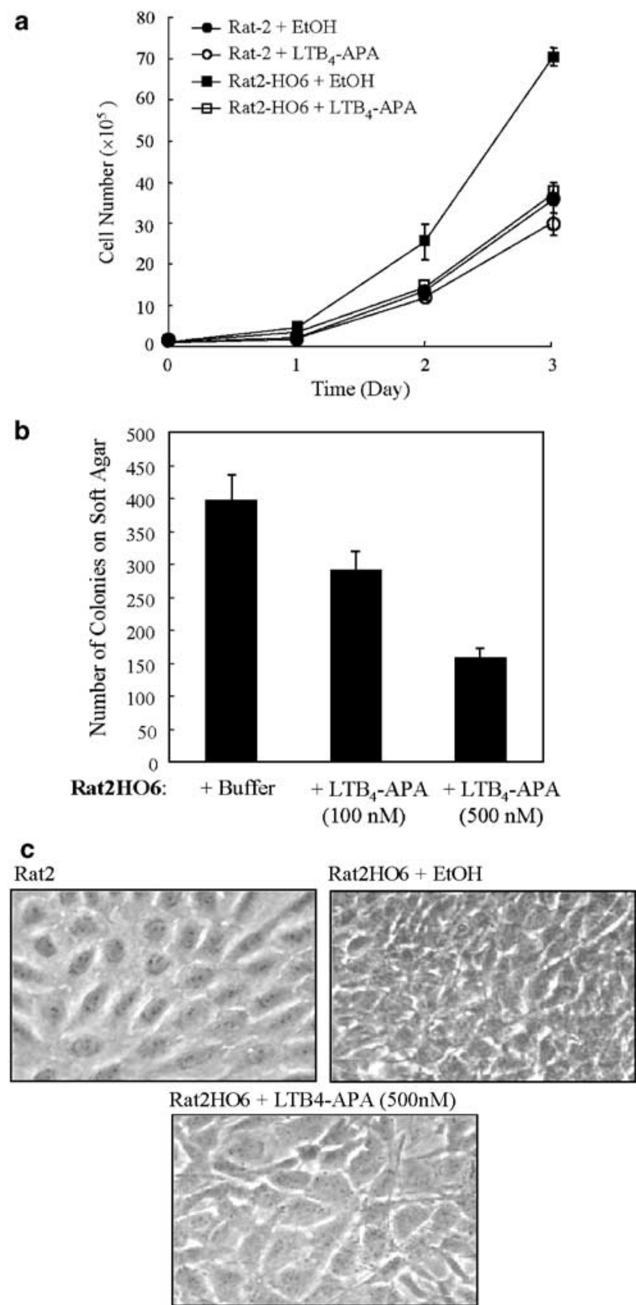

Figure 3 BLT2 blockade suppresses Ha-Ras^{V12}-induced transformation. (**a**, **c**) Inhibitory effect of BLT antagonist LTB_4-APA on Ras transformation. Rat2 and Rat2-HO6 cells were plated to a density of 1×10^5 cells/plate on 60 mm plates. After 12 h, LTB_4-APA (500 nM) or control buffer (EtOH) was added, and cell numbers were counted on the indicated days using the trypan-blue exclusion method (**a**). The corresponding cell morphology was examined and photographed using a phase contrast microscope (**c**). (**b**) Inhibitory effect of LTB_4-APA on anchorage-independent growth of Rat2-HO6. Cells were plated on soft agar, and after 14 days the resultant colonies were stained with *p*-iodonitro tetrazolium violet dye and counted. The experiments summarized in panels a and b were performed in duplicate with two independent sample sets, and the results were averaged

Overexpression of BLT2 induces partial transformation

To confirm the idea that BLT2 is functionally required for Ras-induced transformation, we prepared clones stably overexpressing BLT2 (Rat2/BLT2 cells) and examined the extent to which overexpression of BLT2 affected growth rate, morphology, anchorage-independent growth and smooth muscle α-actin (SMA) promoter activity, which is frequently used as a marker of transformation (Kumar *et al*., 1992). As shown in Figure 4a, the growth rate of Rat2/BLT2 cells was significantly higher than that of control Rat2/pcDNA cells, albeit less than that of Rat2-HO6 cells, and this increase was dramatically attenuated by the BLT antagonist CP105696 (10 μM) (Koch *et al*., 1994; Showell *et al*., 1996; Yokomizo *et al*., 2001a, b) (Figure 4b). Taken together with the aforementioned result showing enhanced production of LTB_4 in stable Rat2-BLT2 cells (Figure 1), these findings suggest that the elevated growth rate seen in Rat2/BLT2 cells is likely elicited by an autocrine action of LTB_4 via BLT2 receptors, which amplifies the LTB_4-dependent cascade. In addition to the elevated growth rate, partial transformed morphology was observed in Rat2/BLT2 cells, although again the change was not as pronounced as in Rat2-HO6 cells (Figure 4c). For example, confluent Rat2/BLT2 cells showed partial loss of contact inhibition, piling up to form multilayers in certain areas, whereas Rat2 and vector-transfected control cells remained as a monolayer with flattened phenotype. In addition, Rat2/BLT2 cells showed some capacity for anchorage-independent growth on soft agar, though once again the effect was not as pronounced as in Rat2-HO6 cells (Figure 4d).

SMA promoter activity is known to be suppressed in transformed cells, an effect reflected by the rounded (as opposed to flattened) shape of transformed cells. When cells were transiently transfected with a plasmid encoding the SMA promoter fused to the luciferase reporter gene, we found the SMA promoter activities in Rat2-HO6 and Rat2/BLT2 cells to be reduced to about 10% of that seen in normal Rat2 or Rat2/pcDNA cells

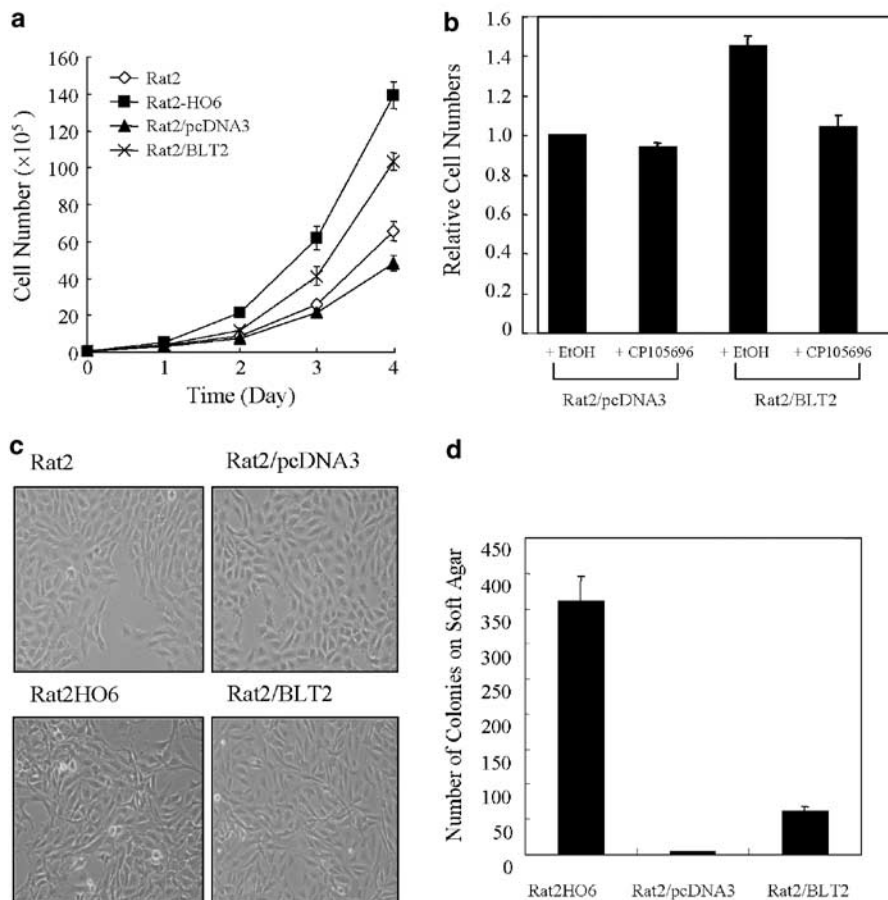

Figure 4 Overexpression of BLT2 elicits partial cell transformation. (**a**) The growth rates of Rat2-HO6, Rat2/pcDNA3 and Rat2/BLT2 cells were evaluated as in Figure 3a. (**b**) Effect of CP105696 (10 μM) on the relative growth rates of Rat2/BLT2 and Rat2/pcDNA3 cells. The cells were counted after 3 days using trypan-blue exclusion method, and the data are expressed as fold of control (Rat2/pcDNA3 cells treated with EtOH), which was assigned a value of 1.0. (**c**) Cell morphology was examined and photographed using a phase contrast microscope. (**d**) Anchorage-independent growth of Rat2/BLT2 cells was evaluated as in Figure 3b. The experiments summarized in panels a and d were performed in duplicate with two independent sample sets, and the results were averaged

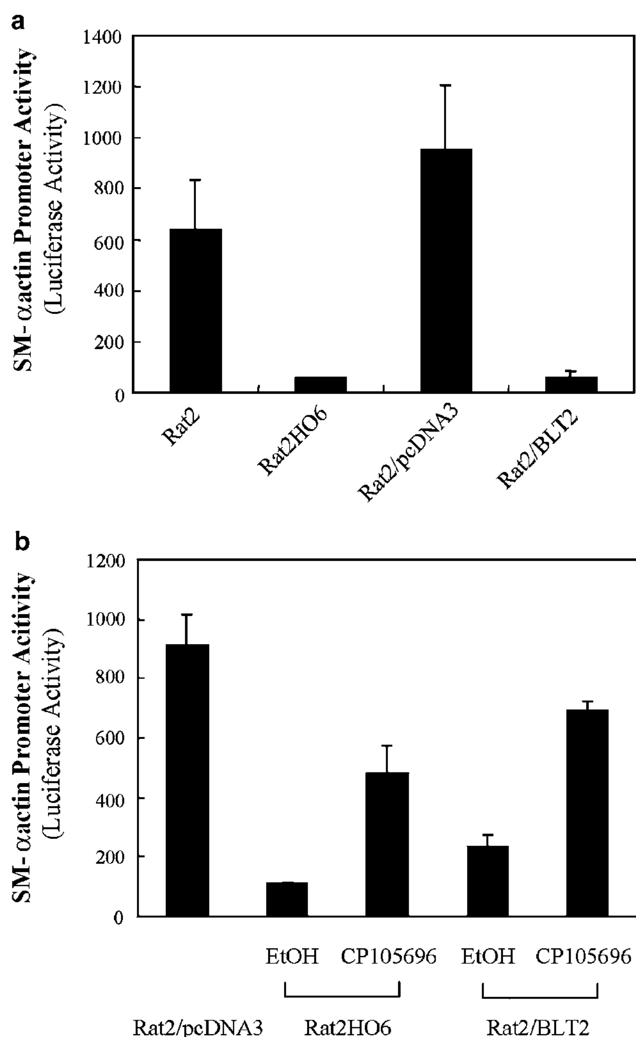

Figure 5 The smooth muscle α-actin promoter is repressed by Ras transformation or BLT2 overexpression. (**a**) Smooth muscle α-actin promoter activity in the indicated cell types is expressed as a function of luciferase reporter activity, which was normalized to β-galactosidase activity and protein concentration. (**b**) Effect BLT2 blockade on smooth muscle α-actin promotor activities in Rat2-HO6 and Rat2/BLT2 cells. The cell were treated as in panel a, except that CP105696 (20 μM) or control vehicle (EtOH) was added for 24 h before harvesting and assaying luciferase activity. All experiments were performed in duplicate with two independent isolated sets, and the results were averaged

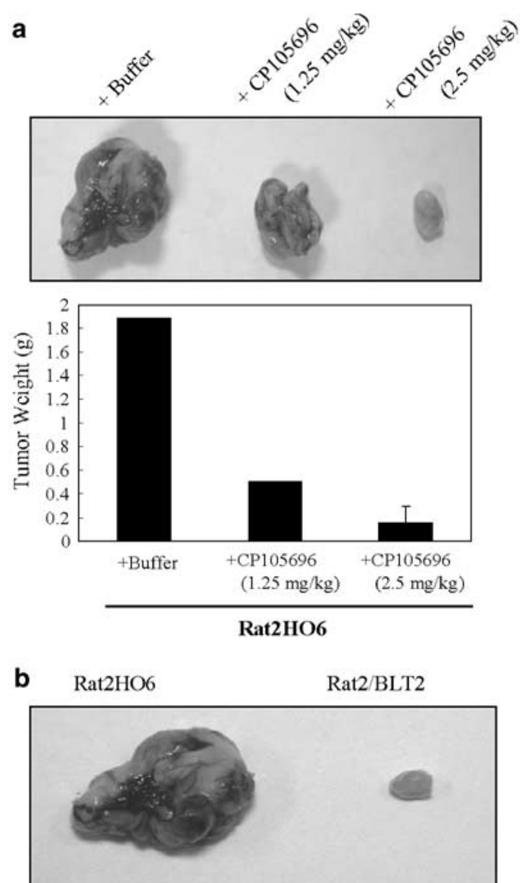

Figure 6 BLT2 antagonist, CP105696, suppressed tumor formation of Rat2-HO6 in athymic mice. (**a**) Effect of CP105696 on the tumor formation by Rat2-HO6 cells. Cells (5×10^5 cells) were subcutaneously injected into the flanks of athymic mice. CP105696 (1.25 and 2.5 mg/kg of weight) or control buffer was then injected intraperitoneally 12, 15 and 18 days later. At 3 weeks after cell injection, the mice were killed and the tumors were removed and analysed. (**b**) Tumorigenicity of Rat2/BLT2 cells. Equal numbers (2×10^6) of Rat2/pcDNA and Rat2/BLT2 cells were subcutaneously injected into athymic mice. After 3 weeks, tumors were removed and photographed. The results shown are representative of three independent experiments with similar results

(Figure 5a), reflecting a transformed phenotype. Moreover, some of the activity could be recovered by blockade of BLT receptors using CP105696 (20 μM for 24 h) (Figure 5b), further confirming the critical involvement of BLT2 in the transformation induced by Ha-Ras^{V12}.

BLT2 blockade suppresses tumor formation in athymic mice

As Ha-Ras^{V12}-transformed cells are known to form tumors in athymic mice, we evaluated the role of BLT signaling in tumorigenicity by subcutaneously injected Rat2 (1.5×10^6 cells), Rat2-HO6 (5×10^5 cells) or Rat2/BLT2 (1.5×10^6) cells into athymic BALB/*c*-nuSlc mice. In addition, CP105696 (1.25 and 2.5 mg/kg body weight) was injected intraperitoneally 12, 15 and 18 days after cell injection. Within 3 weeks, Rat2-HO6 cells formed a large tumor mass, the size of which was dose-dependently diminished by CP105696 (Figure 6a). Tumor weights in CP105696 (2.5 mg/kg)-treated mice were about 10% of those in buffer-treated mice. Tumors also developed in Rat2/BLT2-injected mice, but they were much smaller than those formed by Rat2-HO6 cells (Figure 6b) and Rat2/pcDNA cells showed no ability to form tumors (data not shown). Together, these results suggest that BLT2 blockade could suppress the tumor formation of Ras-transformed cells *in vivo*, consistent with the proposed role of BLT2 as a crucial mediator in the signaling leading to transformation or tumorigenicity induced by oncogenic Ras.

Human tumor tissues show elevated levels of BLT2 mRNA

To further investigate the physiological importance of BLT2 *in vivo*, we measured levels of BLT2 mRNA in various human cancers and corresponding normal tissues. To do this, *in situ* hybridization was carried out using *in vitro*-transcribed probes labeled with digoxigenin (DIG) in a panel of 10 cancers, including skin squamous cell carcinoma, esophagus squamous cell carcinoma, breast ductal carcinoma, colon adenocarcinoma, kidney renal cell carcinoma, bladder transitional cell carcinoma ovary serous carcinoma, thyroid gland follicular carcinoma, uterus cervix carcinoma and lung adenocarcinoma. Most of the cancers showed elevated expression of BLT2 mRNA, as compared to noncancerous tissues. This was particularly true for thyroid gland follicular carcinoma, kidney renal cell carcinoma, bladder transitional cell carcinoma, esophagus squamous cell carcinoma, colon adenocarcinoma, ovary serous carcinoma and uterus cervix carcinoma, which showed greatly increased levels BLT2 mRNA (Figure 7).

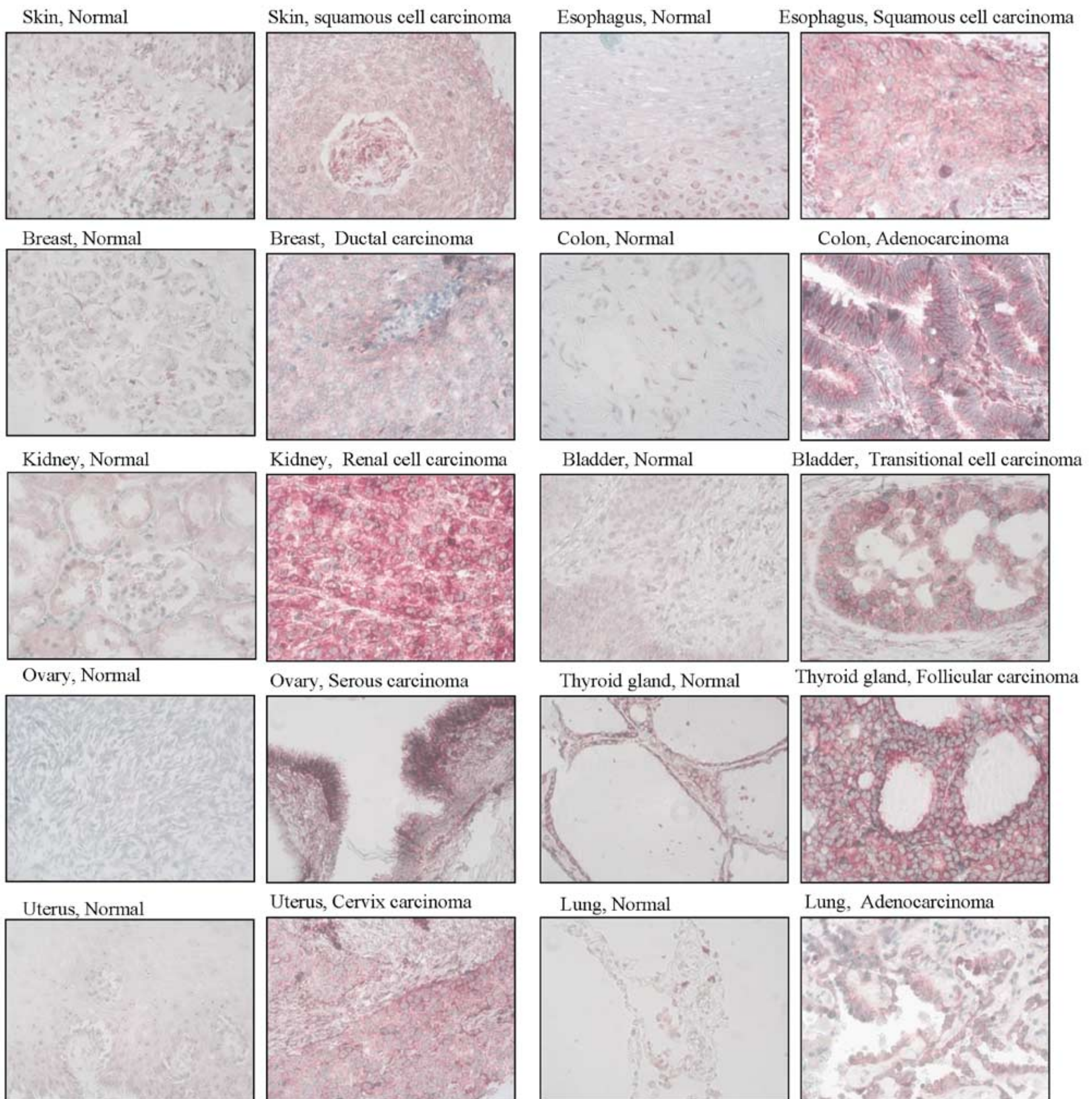

Figure 7 Increased expression of BLT2 mRNA in various human cancers. To evaluate expression of BLT2 mRNA in various cancers and corresponding noncancerous tissues, *in situ* hybridization was carried out using DIG-labeled RNA probes. Human BLT2 plasmids were used for probe preparation, as described in Materials and methods. The results shown are representative of three independent experiments with similar results

Discussion

In the present study, we showed that levels of LTB_4 and LTB_4 receptor-2 (BLT2) are significantly elevated in Ras-transformed cells; that Ras-transformed phenotypes are markedly inhibited by BLT2 blockade, as was their tumorigenicity *in vivo*; that overexpression of BLT2 elicits partial transformation of normal Rat-2 fibroblasts. Further, we presented evidence suggesting that expression of BLT2 is significantly elevated in a variety of human cancers.

The high-affinity LTB_4 receptor BLT1 is known to be expressed exclusively in inflammatory cells, such as leukocytes (Yokomizo *et al*., 1997). To date, most studies of LTB_4 receptors have focused on BLT1, especially on its role in inflammatory processes. In fact, LTB_4 are known to elicit a variety of inflammatory responses, including leukocyte activation, chemotaxis and degranulation (Samuelsson *et al*., 1987; Chen *et al*., 1994), and production of LTs is upregulated in areas of inflammation, leading to tissue hyperplasia and hyper-responsiveness to mediators at the inflammatory site. In addition, overproduction of LTB_4 is now known to be involved in such inflammation-related ailments as bronchial asthma and rheumatoid arthritis (Griffiths *et al*., 1995; Turner *et al*., 1996).

In contrast to BLT1, no clear physiological function has yet been identified for BLT2, which shows a lower affinity for LTB_4, but which is widely expressed in a variety of tissues, with highest levels seen in spleen (Yokomizo *et al*., 2000). The findings of the present study demonstrate for the first time a potential physiological role for BLT2, acting as a downstream mediator of Ras in the pathway to cell transformation. Consistent with LTB_4's proposed function as a mediator in the oncogenic H-Ras^{V12} signaling to transformation, Rat2-HO6 cells showed increased expression of $cPLA_2$, 5-LO and FLAP, three crucial proteins involved in the synthesis of LTB_4 (Figure 2a). In that regard, $cPLA_2$ expression was previously shown to be upregulated in a number of cancer cell lines and to contribute to the induction of a number of transformed phenotypes (Heasley *et al*., 1997; Blaine *et al*., 2001; Yoo *et al*., 2001). In support of our findings, it has also been reported that downmodulation of 5-LO by nonsteroidal anti-inflammatory drugs can reverse carcinogenesis and that inhibition of LTs inhibits vinyl carbamate-induced lung tumors in mice (Shureiqi and Lippman, 2001; Gunning *et al*., 2002). Conversely, LTB_4 appears to simulate cell proliferation, and there is increased secretion of LTs from tumors (Hennig *et al*., 2002; Tong *et al*., 2002). Moreover, LTB_4 receptor was reported to be expressed in human pancreatic cancers, but not in the normal pancreatic tissue (Hennig *et al*., 2002; Tong *et al*., 2002) although the exact role was not characterized.

The contribution made by LTB_4 to cell proliferation or malignant transformation appears to be much more widespread than first suspected, and our results showing elevated BLT2 mRNA levels in various human cancer tissues (Figure 7) are in good agreement with earlier reports suggesting *in vivo* involvement of *ras* in carcinogenesis – for example, activated Ha-*ras* gene mutation is frequently seen in bladder cancer, skin cancer, thyroid cancer and squamous cell carcinoma (Corominas *et al*., 1991; Capella *et al*., 1996; Kreimer-Erlacher *et al*., 2001). Interestingly, cells overexpressing BLT2 show higher levels of LTB_4 production than wild-type cells (Figure 1), which suggests that there could be a regulatory amplifying cross-talk between LTB_4 and BLT2 such that each induces the expression of the other. Indeed, similar cross-talk among eicosanoid lipid ligands and their receptors has been described previously (Dohadwala *et al*., 2002).

AA released from membrane phospholipids is known to be metabolized to LTs by LOs and to prostanoids by COXs. COXs are well known to be involved in carcinogenesis – for example, cellular expression of COX-2 is induced by the viral oncogene *v-src* (Kujubu *et al*., 1991; Xie and Herschman, 1995), and their expression has been detected in a variety of cancers (Buckman *et al*., 1998; Bauer *et al*., 2000; Bae *et al*., 2001; Kundu *et al*., 2001; Patti *et al*., 2002). For that reason, several attempts have been made to slow cancer growth by inhibiting COX-2 (Fournier and Gordon, 2000; Abou-Issa *et al*., 2001; Dang *et al*., 2002). In the present study, however, we observed no involvement of COXs or prostanoids in Ha-Ras^{V12}-induced cell transformation. Pretreating Rat2-HO6 cells with indomethacin, a nonspecific COX inhibitor, had no effect on transformation phenotypes (data not shown), and levels of COXs proteins and prostanoids were unchanged in Rat2-HO6 cells (data not shown), excluding the involved roles of COXs in the signaling to transformation, at least, induced by oncogenic H-Ras.

Although the results presented make the critical involvement of an LTB_4-BLT2-linked cascade in Ras-mediated transformation apparent, the signaling pathway from BLT2 stimulation to transformation remains totally unknown. Previously, we reported that exogenous LTB_4 induces BLT-dependent chemotactic migration in both Rat-2 and neutrophil cells, and that LTB_4-evoked chemotaxis could be blocked by inhibiting ROS generation, thereby implicating ROS in the LTB_4-BLT2 signaling to migration (Woo *et al*., 2002, 2003). We therefore hypothesize that ROS generation may lie downstream of BLT2 in the signaling pathway from oncogenic Ha-Ras^{V12} to cell transformation. Our prediction that ROS serves as a downstream mediator of BLT2 in the Ras-transformation pathway is in good agreement with the findings of Goldschmidt-Clermont *et al*., who reported that ROS generated by Ha-Ras^{V12} somehow mediate oncogenic signaling in fibroblasts. They proposed that Rac, but not the Raf-MAPK-linked cascade, is involved in the signaling to ROS generation, thereby mediating Ha-Ras^{V12} signaling to cell transformation (Irani *et al*., 1997). A similar conclusion was drawn by Yang *et al*. (2002), who found that overexpression of superoxide dismutase inhibited Ras-induced transformation, although the signaling mechanism for the enhanced ROS generation in Ras-transformed cells was not characterized.

This is the first report on the relationship between Ras signaling and LTB_4-BLT2-linked cascade, and the first demonstration of a physiological function of BLT2. Considering the well-known role of LTB_4 in inflammation, our study will provide a valuable basis for a new line of research into the interrelated mechanisms of LTB_4 signaling, inflammation and Ras transformation. Beyond that, the finding that an LTB_4-BLT2-linked cascade is crucially involved in the signaling for Ras-induced transformation both *in vitro* and *in vivo* could potentially serve as the basis for the development of new diagnostic tools and therapeutic strategies for the treatment of human cancer.

Materials and methods

Cell culture

Rat2 and Rat2-HO6 (Kumar *et al.*, 1992) cells were maintained in Dulbecco's modified Eagle medium (DMEM; Life technologies, Inc.) supplemented with 0.1 mM MEM nonessential amino acids (Life technologies, Inc.), 10% fetal bovine serum (FBS; Life technologies, Inc.), and antibiotic–antimycotic solution (Life technologies, Inc.) at 37°C under a humidified atmosphere of 5% CO_2. Cells were maintained in a growth phase by splitting them every 3 days using Trypsin-EDTA, after which they were washed in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) prior to adding new medium.

To prepare stable Rat2/BLT2 clones, Rat-2 cells were transiently transfected with $pcDNA_3$-BLT2 (Yokomizo *et al.*, 2000) encoding HA-tagged human BLT2 and then selected with 0.4 mg/ml of G418 (Invitrogen, Carlsbad, CA, USA). The selected clones were screened for BLT2 expression by RT–PCR using human-specific BLT2 primers (see below – *RT–PCR of BLT2*), and a representative clone was used for experimentation.

LTB_4 quantifications and growth assays

To measure the LTB_4 levels, Rat2-HO6 and Rat2/BLT2 cells (5×10^5/plate) were plated on 60 mm culture dishes and incubated in DMEM supplemented with 10% FBS for 24 h. The culture medium was then replaced with DMEM containing 0.5% FBS and incubated for an additional 24 h, after which the conditioned medium was collected and immediately frozen. The collected medium was then lyophilized, and LTB_4 was quantified using a specific ELISA as instructed by the manufacturer (Amersham Pharmacia Biotech, Ltd, UK). The ELISA was calibrated with LTB_4 standards ranging from 0.31 to 40 pg/well. Statistical significance of differences between groups was assessed with analysis of variance (ANOVA; $P < 0.01$). To test the effects of LT receptor inhibitors LTB_4-APA (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA, USA) and CP105696 (kindly provided by Pfizer Inc., Groton, CT, USA) on cell proliferation, 1×10^5 cells were seeded onto 60 mm culture dish and allowed to adapt for 12 h. LTB_4-APA (500 nM) or CP105696 (10 μM) was then added to the cultures for 24, 48 or 72 h, after which cell morphology was examined under a phase-contrast microscope, after which the cells were harvested and counted using the trypan-blue exclusion method.

Western blot analysis

To determine the expression levels of $cPLA_2$, 5-LO and FLAP, Rat-2 and Rat2-HO6 cells (5×10^5 cells/dish) were seeded onto 60 mm culture dishes and allowed to adapt for 24 h. They were then washed with ice-cold PBS, and whole-cell lysates were obtained using lysis buffer (20 mM Tris–Cl, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 10 mM NaF, 5 mM EGTA, 0.5 mM PMSF, 5 μg/ml leupeptin and aprotinin, 3 mM DTT). The protein content was quantified using Bradford reagent, after which 20-μg samples were subjected to SDS–PAGE, and the separated proteins were transferred to a PVDF membrane (Amersham Pharmacia Biotech, Ltd, UK) using a wet transfer system (Bio-Rad, Hercules, CA, USA). The membrane was then blocked in 5% skim milk in TBST (0.05% Tween-20, 25 mM Tris–Cl (pH 7.4), 150 mM NaCl), washed twice with TBST for 10 min each, and incubated first with the primary antibody (anti-5-LO, anti-FLAP or anti-$cPLA_2$ antibody) in blocking buffer for 3 h at room temperature, and then with HRP-conjugated secondary antibody (New England BioLabs Inc., Beverly, MA, USA). After washing with TBST, the blots were incubated with ECL solution (Amersham Pharmacia Biotech, Ltd, UK) and exposed to X-ray film.

RT–PCR of BLT2

After plating Rat2 and Rat2-HO6 cells (8×10^5 cells/dish) on 60 mm culture dishes and allowing them to adapt for 24 h, samples of RNA were isolated from the cells using Trizol B RNA extraction reagent (Invitrogen, Inc., Carlsbad, CA, USA). Thereafter, 1 μg of total RNA was reverse transcribed for 1 h at 42°C and amplified by PCR using specific primers (5′-GCATGTCCCTGTCTCTGTTG-3′ (sense rat BLT1); 5′-CGGGCAAAGGCCTTAGTACG-3′ (antisense rat BLT1); 5′-GCTACTCTGACATCGGGCGC-3′ (sense rat BLT1); 5′-GGAAGTCATGAAGCTGTCGG-3′ (antisense rat BLT1); 5′-CAGCATGTACGCCAGCG TGC-3′ (sense rat BLT2); 5′-CGATGGCGCTCACCAGACC-3′ (antisense rat BLT2)) from Genotech Inc. (Korea). The PCR protocol entailed 25 cycles of 94°C for 1 min and 68°C for 2 min, followed by a final extension at 72°C for 10 min. PCR products were separated by electrophoresis on 1.2% agarose gels and stained with ethidium bromide. GAPDH 5′-CTGCACCACCAACTGCT-TAGC-3′ (sense); 5′-CTTCACCACCTTC TTGATGTC-3′ (antisense); served as an internal quantitative control.

Soft agar assay

A total of 1000 Rat2, Rat2-HO6 and Rat-BLT2 cells suspended in 6 ml of 0.35% noble agar (growth medium with 10% FBS) containing LTB_4-APA or buffer were poured onto the 100 mm plates masked with a 6 ml basal layer of 0.7% noble agar in DMEM. The plates were then incubated in a humidified 37°C incubator for 14 days, pouring growth medium onto the agar plate every 7 days. The colonies that developed were visualized by staining with *p*-iodonitro tetrazolium violet (INT); Sigma, St Louis, MO, USA) overnight and counted.

Smooth muscle α-actin promoter assay

To assess smooth muscle α-actin promoter activity, cells (5×10^5 cells/100 mm dish) were transiently transfected using the calcium phosphate method. Briefly, calcium phosphate : DNA precipitates were prepared with a total of 20 μg of DNA, including 15 μg of pαAluci reporter plasmid (Kumar *et al.*, 1992). To control for variations in cell number and transfection efficiency, all cells were co-transfected with 1 μg of pSV-β-

gal, a eucaryotic expression vector in which the *Escherichia coli* β-galactosidase (lac Z) structural gene is under the transcriptional control of the SV40 promoter. The total amount of DNA in each transfection was kept constant at 20 μg by adding appropriate amounts of sonicated calf thymus DNA (Sigma). After incubating for 6 h with the calcium phosphate : DNA precipitates, the cells were rinsed twice with PBS and then incubated in fresh DMEM supplemented with 0.5% FBS for an additional 48 h. Thereafter, cell extracts were prepared by rinsing each plate twice with PBS and lysing the cells in 0.2 ml of lysis solution (25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 0.1% Triton X-100). The lysed cells were scraped and spun for 1 min, after which the supernatants were assayed for protein content and luciferase and β-galactosidase activities.

Luciferase activity was assayed in 10-μl samples of extract using a luciferase assay system according to the manufacturer's (Promega, Madison, WI, USA) protocol. Luciferase luminescence was counted in a luminometer (Berthold technologies, Ltd, Germany) and normalized to cotransfected β-galactosidase activity. β-Galactosidase assays were carried out using 100 μl of extract and 100 μl of 2 × reaction buffer (1.5 mg/ml *O*-nitrophenyl-β-galactopyranoside, 2 mM $MgCl_2$, 61 mM Na_2HPO_4, 39 mM NaH_2PO_4, 100 mM 2-mercaptoethanol). When a faint yellow color appeared, the reactions were stopped by addition of 350 ml of 1 M Na_2CO_3, and absorbance at 410 nm was measured in a spectrophotometer. The results were then used to normalize luciferase activity to transfection efficiency. Protein concentrations were routinely measured using the Bradford procedure with Bio-Rad dye reagent (Bio-Rad), with BSA serving as a standard. Transfection experiments were performed in duplicate with two independently isolated sample sets, and the results were averaged.

Tumor formation assay

Female 5-week-old athymic mice (BALB/*c*-nuSlc) were purchased from Japan SLC Inc. and housed under specific pathogen-free conditions for 1 week. Rat2 and Rat2-HO6 cells (5×10^5 cells) maintained in the growth phase were subcutaneously injected into the flanks of the athymic mice. For inhibitor experiments, CP105696 (1.25 or 2.5 mg/kg of weight) was injected intraperitoneally 12, 15 and 18 days after injection of the cells. At 3 weeks after injection, the mice were killed and the tumors were removed and analysed. To evaluate the tumorigenicity of Rat2/BLT2 cells, equal numbers (2×10^6) of Rat2/pcDNA or Rat2/BLT2 cells were injected into the athymic mice as above.

In situ *hybridization for BLT2*

For the preparation of an antisense probe for BLT2 mRNA, the human BLT2 expression plasmid pcDNA3-BLT2 (kindly provided by Dr Takao Shimizu of Tokyo University, Tokyo, Japan) was modified using pcDNA3(−) vector to prepare pcDNA3-reverseBLT2, which was confirmed by DNA sequencing. pcDNA3-reverseBLT2 was linearized using *Afe*I restriction endonuclease (MBI Fermentas Ltd), after which the linearized vectors were transcribed using T7 RNA polymerase and DIG (digoxigenin) RNA labeling mix (Roche, Germany). The transcribed probe was ethanol precipitated and quantified by measuring the absorbance at 260 nm. Various cancer tissue samples plus matching normal tissue arrays from Petagen Inc. (Korea) were deparaffinized with xylene, after which *in situ* hybridization was carried out using an *in situ* hybridization detection kit according to manufacturer's protocol (InnoGenex, San Ramon, CA, USA). Briefly, deparaffinized tissues were treated with Proteinase K and post-fixed with 1% formaldehyde in RNase-free PBS. After hybridizing the DIG-labeled probes for 16 h at 37°C, they were reacted with anti-DIG antibodies, and aminoethyl carbazole (AEC) reagent was used for color development. Mayer's hematoxylin served as the counter staining.

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