# Platelet-type 12-lipoxygenase accelerates tumor promotion of mouse epidermal cells through enhancement of cloning efficiency

# Platelet-type 12-lipoxygenase accelerates tumor promotion of mouse epidermal cells through enhancement of cloning efficiency

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Running title: *p*12-LOX in tumor promotion

Key words: lipoxygenase, tumor promotion, chemoprevention, JB6

Abbreviations: COX, cyclooxygenase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HETE, hydroxyeicosatetraenoic acid; LOX, lipoxygenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide; NDGA, nordihydroguaiaretic acid; *p*12-LOX, platelet-type 12-lipoxygenase; PG, prostaglandin; RT-PCR, reverse transcription-polymerase chain reaction; siRNA; small interfering RNA; TNF-α, tumor necrosis factor-α, TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

**Abstract**

Accumulating evidence suggests that platelet-type 12-lipoxygenase (*p*12-LOX) plays an important role in tumor development. However, how *p*12-LOX contributes to tumorigenesis is still not understood yet. The role of *p*12-LOX was therefore examined in tumor promotion using mouse epidermal JB6 P+ cells that are sensitive to 12-*O*-tetradecanoylphorbol-13-acetate(TPA)-induced transformation. The expression of *p*12-LOX was significantly higher in JB6 P+ cells than in JB6 P– cells that were resistant to transformation, and its expression was further increased by tumor necrosis factor (TNF)-α. Importantly, the inhibition of *p*12-LOX in JB6 P+ cells by baicalein, a specific inhibitor or small interfering RNA significantly suppressed TPA-induced transformation. Moreover, treatment with 12(*S*)-hydroxyeicosatetraenoic acid (HETE), a metabolite of *p*12-LOX, enhanced TPA-induced neoplastic transformation either in the presence or absence of baicalein. These results indicate that *p*12-LOX is required for tumor promotion of epidermal cells and that 12(*S*)-HETE functions as a rate-limiting factor. Notably, treatment with baicalein significantly suppressed the proliferation of JB6 P+ cells when cells were seeded at a low density in a culture plate. Moreover, the cloning efficiency of JB6 P+ cells was dramatically decreased by inhibition of *p*12-LOX. In contrast, baicalein treatment did not affect the cloning efficiency of most malignant cancer cells. These results indicate that *p*12-LOX is induced by the inflammatory cytokine TNF-α in the early stage of tumorigenesis, and is required for tumor promotion through enhancing efficient proliferation of a small number of initiated cells. The present results suggest that the *p*12-LOX pathway may be an effective target of chemoprevention for skin carcinogenesis.

**Introduction**

The cyclooxygenase (COX) and lipoxygenase (LOX) are two important enzyme classes that metabolize polyunsaturated fatty acids and play important roles in tumorigenesis (1). COX-2, one of the COX isoenzymes, plays a variety of pathological roles including cancer development (2). Previous studies have demonstrated that the induction of COX-2 is responsible for intestinal tumorigenesis (3) and that prostaglandin (PG)$E_2$, one of downstream products of COX-2, is required for carcinogenesis in the stomach through cross-talk with activated Wnt signaling (4,5).

Among the LOX family members, it has been suggested that platelet-type (*p*12-LOX) plays an important role in tumorigenesis (6,7). The expression of *p*12-LOX is absent or at a basal level in the normal tissues (6,8). However, the induction of *p*12-LOX is widely found in human cancer tissues that develop in the colon (9), prostate (10,11), stomach (12), and skin (13). In animal models, the *p*12-LOX expression is also found in xenografts of melanoma (9) and in skin tumors developed by an initiation/promotion protocol (14). Moreover, it has been shown that *p*12-LOX expression is associated with the advanced stages of human prostate cancer and its levels increase in metastasized tumors in a mouse model (10,11). These analyses suggest that *p*12-LOX contributes to development and progression of various types of cancers.

The treatment of cancer cells with baicalein, a *p*12-LOX inhibitor, has also been shown to result in the induction of apoptosis and inhibition of tumor xenograft growth in immunodeficient mice (13,15-17). Consistently, the forced expression of the *p*12-LOX gene or stimulation with 12(*S*)-HETE, a metabolite of *p*12-LOX, has been shown to induce cell proliferation and survival (16,18,19). Moreover, it has been reported that *p*12-LOX induces cell spreading, integrin-dependent cell survival (19,20) and angiogenesis through the induction of vascular

endothelial growth factor (VEGF) expression (21,22). Accordingly, it is conceivable that the *p*12-LOX pathway also contributes to tumor progression through the acceleration of cell survival, migration and angiogenesis.

Genetic experiments have indicated that *p*12-LOX plays an important role in skin tumorigenesis. In the initiation/promotion skin carcinogenesis model, multiplicity and incidence of squamous cell carcinoma are suppressed significantly by the disruption of the *p*12-LOX gene (23). Accordingly, it is possible that *p*12-LOX is important for tumor promotion of the initiated cells during skin carcinogenesis. To assess this possibility, we have investigated the role of *p*12-LOX and 12(*S*)-HETE in promotion of epidermal cell transformation using mouse JB6 P+ cell line. The cells of this line are transformed by stimulation with tumor promoters such as TPA or TNF-α, and therefore, are useful to examine the mechanisms involved in the promotion of epidermal cell transformation (24,25). Clonal variant JB6 P– cells are resistant to tumor promoter-induced transformation. As a result, we therefore used JB6 P– cells, in addition to P+ cells, to examine the role of *p*12-LOX in the transformation process.

We demonstrate here that expression of *p*12-LOX is increased significantly in JB6 P+ cells but not in JB6 P– cells. Moreover, inhibition of *p*12-LOX of JB6 P+ cells suppresses colony formation in soft agar, while treatment with 12(*S*)-HETE accelerates it. In addition, we show that the *p*12-LOX pathway is responsible for proliferation from a single cell or a small number of cells. These results suggest that *p*12-LOX contributes to tumor promotion through the acceleration of initiated cell proliferation, and that lack of *p*12-LOX is thus considered to be one of the causes for the resistance of JB6 P– cells against tumor promoter-induced transformation.

**Materials and methods**

*Reverse transcription–polymerase chain reaction analysis*

RNA samples were prepared from cultured cells or mouse tissue samples using the ISOGEN solution (Nippongene, Tokyo, Japan). Mouse gastric tumors and intestinal polyps were obtained from *K19-Wnt1/C2mE* mice (6) and $Apc^{\Delta 716}$ mice (26), respectively. Control tissues were obtained from normal tissues of the same mice or wild-type mice. Extracted RNA was reverse-transcribed and amplified by PCR with the following primer set: *p*12-LOX (F-5'-GCGGTCTTCGAATTGAACTT-3', and R-5'-CAGGAACAGTGTTGGAGCTG-3'); epidermal-type 12-LOX (F-5'-CAGGAGCTGGAGAACAGAAGG-3', and R-5'-GAACTGGTACCCAAAGAGAGCATC-3'); leukocyte-type 12-LOX (F-5'-GGCTCCAACAACGAGGTCTA-3', and R-5'-AGTTCCTCCTCCCTGTGGTT-3'); 5-LOX (F-5'-AATGGAGGTGGTGAGCATCTA-3', and R-5'-TCCTTCACTGGCTTCTCAATG-3'); 8-LOX (F-5'-CGAAATGCAGGGTGAGAGTA-3', and R-5'-TCCTGCAGTGTAGGGTGATG-3'). Animal experiments were carried out according to the protocol approved by the Committee on Animal Experimentation on Kanazawa University.

*Cell culture and soft agar colony formation assay*

JB6 P+ (Cl 41-5a) and JB6 P– (Cl 30-7b) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in MEM supplemented with 5% FBS and incubated in a 5% $CO_2$ at 37$^{o}$C. For the soft agar colony formation assay, JB6 P+ and P– cells were exposed to TPA (Calbiochem, San Diego, CA) at 10 ng/ml in 1 ml of basal medium eagle

(Invitrogen, Grand Island, NY) with 10% FBS and 0.33% agar, and then seeded in a 6-well plate ($10^4$ cells/well). Soft agar cultures were maintained in a 5% $CO_2$ incubator at 37°C for 14 days, stained with Giemsa stain solution (Wako, Osaka, Japan), and the number of colonies was scored using a stereomicroscope and Image J software program (NIH). A nonselective LOX inhibitor NDGA, a selective 12-LOX inhibitor baicalein (Tocris Biosciences, Ellisville, MI), or a selective 5-LOX inhibitor AA-861 (Sigma, St. Louis, MO) was added to the agar containing medium at 5 μM (NDGA and baicalein) or 10 μM (AA-861) to test their inhibitory effect on anchorage independent transformation. To examine the effect of 12(*S*)-HETE, cells were pre-treated with 12(*S*)-HETE (Cayman Chemical, Ann Arbor, MI) at 0.1 μM or 1 μM for one day prior to stimulation with TPA, and were plated as described above for the soft agar colony formation assay. All soft agar colony formation assays were repeated three times, and the mean colony numbers and S.D. were calculated.

*Subcutaneous transplantation of tumor cells to mice*

JB6 P+ cells were stimulated with TPA at 10 ng/ml for 12 h and inoculated *s.c.* at $1.0 \times 10^6$ cells/site to 3 sites per mouse for four mice each for drug-treated and no-treated group (Balb/c-nu/nu: Charles River Laboratories Japan, Tokyo, Japan). At 5 days after inoculation, 50 μl of TPA (10 ng/ml in PBS) was injected *s.c.* into the inoculation site. After palpable tumors developed, baicalein was administered at 20 mg/kg/day in 0.5% methylcellulose (Wako) *p.o.*, and treatment was continued for 14 days. In another dosing experiment, baicalein treatment started from day 7 after cell inoculation, and the treatment was continued until 1 week after palpable tumors developed in the control mice. The dosing protocol has been reported to inhibit *p*12-LOX activity

*in vivo* (27). The tumor size was measured every 2 days during drug treatment using 6 developed tumors for each experiment and the mean tumor volume and standard error were calculated.

*BrdU labeling analysis*

Mice were injected *i.p.* with 100 μl of BrdU solution (BD Pharmingen, San Diego, CA) at 1 h before euthanasia. The tumor tissues were fixed in 4% paraformaldehyde, embedded and sectioned at 4-μm thickness. These sections were stained with anti-BrdU antibody (BD Pharmingen). The immunostaining analysis was repeated three times.

*Small interfering RNA transfection*

The target sequence of three small interfering RNAs (siRNAs) against *p*12-LOX were: A, sense: GGGUGCAGGGAGAGGGAAUTT, antisense: AUUCCCUCUCCCUGCACCCTT; B, sense: GGAUGGAAUUCCAGCUAAUTT, antisense: AUUAGCUGGAAUUCCAUCCTT; and C, sense: CAUCUCAGAUGGAGGAAUATT, antisense: UAUUCCUCCAUCUGAGAUGTT. We confirmed the siRNA sequences to be specific for mouse *p*12-LOX by GenBank database search. There is no transcript in the database which has >80% homology with siRNA sequences except for *p*12-LOX. These three siRNAs (A, B, and C) were mixed and transfected into JB6 P+ cells at 50 nM using siFECTOR (B-Bridge, Sunnyvale, CA. USA) prior to TPA stimulation. A decrease of *p*12-LOX mRNA by siRNA transfection was confirmed by RT-PCR and immunoblotting analysis.

*Immunoblotting analysis*

Cells were collected in buffer and sonicated. After centrifugation at 20,000g, 20 μg of the supernatant was separated in a 10% sodium dodecy sulfate polyacrylamide gel. Antibody for 12-LOX (Santa Cruz Biotechnology, Carlsbad, CA, USA) was used as the primary antibody. The ECL detection system (Amersham Biosciences, Buckinghamshire, UK) was used to detect the specific signals.

*NF-κB activation assay*

JB6 P+ and JB6 P– cells were stimulated with TNF-α (Calbiochem) at 10 ng/ml in the presence or absence of baicalein (Tocris Biosciences) at 5 μM, and the nuclear extract of these cells was prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (PIERCE, Rockford, IL). Activity of NF-κB was measured using NF-κB p65 Transcription Factor Assay Kit (Active Motif, Carlsbad, CA).

*Cell proliferation assay*

The cell proliferation rate was determined using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. Briefly, $10^2$ or $10^3$ cells were plated to each well of a 96-well microplate, cultured overnight, and the cells of the drug-treated group were treated with NDGA (5 μM) or baicalein (5 μM). Following the drug-exposure period (4 or 6 days), 10 μl of MTT (5 mg/ml in PBS) was added to each well, and then the cells were cultured further for 4 h. The formazan crystals that were produced by these cultures were solubilized using 100 μl of isopropyl alcohol containing hydrochloric acid at 40 mM, and the absorbance at 570 nm was measured. All experiments were repeated three times and the mean relative MTT assay results to the no-drug control groups were then calculated and presented.

*Cloning efficiency assay*

JB6 P+ cells, two gastric cancer cells: AGS (American Type Culture Collection) and Kato-III (Cell Resource Center for Biomedical Research, Tohoku Univ., Japan), and three colon cancer cells: HCT-116 (ATCC), DLD-1 and SW480 (Cell Resource Center for Biomedical Research) were used for the cloning efficiency experiments. The gastrointestinal cancer cells were maintained in Roswell Park Memorial Institute (AGS, Kato-III and DLD-1) or Dulbecco's modified Eagle's medium (SW480 and HCT-116) supplemented with 10% fetal bovine serum and cultured in a 5% $CO_2$ incubator at 37$^{o}$C. The cells were suspended in the medium at 10 cells/ml, and 100 μl was dispensed to each well of 96-well microplate to expect plating a single cell in each well. To examine the effect of *p*12-LOX on cloning efficiency, siRNA for *p*12-LOX was transfected prior to TPA stimulation, or baicalein was added to the medium at 5 μM. After culture for 14 days, all wells were stained with Giemsa stain solution (Wako). All experiments were repeated three times and the cloning efficiency was calculated by dividing the number of wells containing proliferating cells with the total number of cell-plated wells.

*Statistical analysis*

The statistical significance of differences was determined by Student's *t*-test. Differences with a value of $P < 0.05$ were considered to be significant.

**Results**

*Increased expression of p12-LOX in promotion-sensitive JB6 P+ cells*

The expression level of *p*12-LOX was first examined by RT-PCR in the promotion-sensitive JB6 P+ cells as well as in promotion-resistant JB6 P– cells. The level of *p*12-LOX mRNA in the JB6 P+ cells was significantly higher than that in the JB6 P– cells (Figure 1A). Stimulation with TPA at 10 ng/ml increased the *p*12-LOX expression level in JB6 P– cells, although its highest level at 12 h was lower than that of the basal level of JB6 P+ cells. In contrast, *p*12-LOX in the TPA-stimulated JB6 P+ cells was increased only slightly possibly caused by constitutive elevation of the basal level. No significant differences were observed in either the expression of other LOX family members, such as epidermal-type (*e*)12-LOX, leukocyte-type (*l*)12-LOX, 5-LOX, and 8-LOX between JB6 P+ cells and JB6 P– cells (Figure 1A).

*Suppression of anchorage independent transformation of JB6 P+ cells by inhibition of p12-LOX*

TPA treatment induced soft agar colony formation of JB6 P+ cells but not of JB6 P– cells that are a resistant line to tumor promoter-induced transformation (Figure 1B). When JB6 P+ cells were treated with a non-specific LOX inhibitor NDGA at 5μM, the number of soft agar colonies decreased significantly by 88%. Moreover, treatment with baicalein, a *p*12-LOX specific inhibitor, at 5 μM also suppressed colony formation in soft agar of JB6 P+ cells by 81% (Figure 1B and C). It has been suggested that 5-LOX is also procarcinogenic lipoxygenase among the LOX family members (1,6). However, treatment with AA-861, a 5-LOX specific inhibitor, at 10 μM did not reduce the number of soft agar colonies (Figure 1B and C). These results indicate that *p*12-LOX but not 5-LOX plays an important role in the neoplastic transformation of JB6 P+ cells.

Baicalein has been reported to be a potent inhibitor of both reticulocyte 15-LOX and *p*12-LOX (28). Therefore, the effects of *p*12-LOX inhibition on soft agar colony formation were examined using a specific siRNA. Transient transfection of siRNA against the *p*12-LOX gene reduced the mRNA level in comparison with that of control cells (Figure 1D). We also confirmed the protein level of *p*12-LOX to have decreased in the siRNA transfected JB6 cells (Figure 1E). Importantly, the inhibition of *p*12-LOX in JB6 P+ cells by siRNA transfection caused a significant suppression of TPA-induced neoplastic transformation by 61% compared with that in the control cells (Figure 1C). Therefore, the siRNA experiments confirmed the role of *p*12-LOX in promotion step of JB6 P+ cell transformation.

*p12-LOX independent growth of TPA-induced JB6 P+ tumors in nude mice*

We examined the possibility that *p*12-LOX is required for the *in vivo* growth of tumor tissues consisting of JB6 P+ cells. TPA-stimulated JB6 P+ cells formed palpable tumors in nude mice at 22 days after subcutaneous inoculation (Figure 2A). In contrast to the anchorage independent transformation assays, treatment of the mice with baicalein at 20 mg/kg/day from day 26 to 40 (14 days) did not suppress tumor growth (Figure 2A and B). There was no significant difference in the BrdU incorporation in tumor cells between baicalein-treated and non-treated mouse tumors (Figure 2C). Moreover, tumor development was not suppressed when the baicalein treatment started from day 7 after tumor cell inoculation (Figure 2D). These observations suggest that *p*12-LOX is less important for the JB6 tumor growth *in vivo* which develops from $10^6$ inoculated cells. Rather, *p*12-LOX appeared to play an important role in the proliferation from a single JB6 cell (*see below*).

*Acceleration TPA-induced transformation of JB6 P+ cells by 12(S)-HETE treatment*

We next examined the role of 12(*S*)-HETE, a metabolite of *p*12-LOX, in tumor promotion. JB6 P+ cells were pre-treated with 12(*S*)-HETE and the TPA-induced soft agar colony formation in the presence or absence of baicalein was examined. Notably, the treatment of JB6 P+ cells with 12(*S*)-HETE reversed the inhibitory effects of baicalein on TPA-induced colony formation in a dose-dependent manner (Figure 3A and B), thus indicating that 12(*S*)-HETE is responsible for tumor promotion of JB6 P+ cells.

Importantly, even in the absence of baicalein, the number of TPA-induced soft agar colonies of JB6 P+ cells was significantly increased by treatment with 12(*S*)-HETE in a dose-dependent manner (Figure 3A and B). In contrast, 12(*S*)-HETE treatment alone did not induce the soft agar colony formation of the TPA-untreated JB6 P+ cells. These results, taken together, indicate that 12(*S*)-HETE is a rate-limiting factor for the promotion of JB6 P+ cell transformation, although stimulation by a tumor promoter, such as TPA, is still necessary for such promotion.

*Induction of p12-LOX by TNF-α stimulation*

JB6 P+ cells are also transformed by TNF-α stimulation, and TNF-α-activated NF-κB is important for neoplastic transformation of JB6 P+ cells (29,30). Stimulation with TPA, acting through the MAP kinase pathway, activates AP-1 and subsequently NF-κB in JB6 P+ cells (31). Therefore, we examined the possibility that *p*12-LOX is involved in activation of NF-κB in the TNF-α-stimulated JB6 P+ cells. Consistent with the findings of a previous report, the treatment

of JB6 P+ cells with TNF-α significantly increased the NF-κB activity (Figure 4A). Notably, the treatment of the cells with baicalein did not affect the NF-κB activity in the TNF-α-stimulated cells, indicating that the *p*12-LOX pathway is not required for the activation of NF-κB. Although NF-κB was activated by TNF-α in JB6 P– cells, the level was significantly lower than that in JP6 P+ cells (Figure 4A). We also confirmed that the TNF-α-induced NF-κB activation in JB6 P– cells was not suppressed by the baicalein treatment. In contrast, stimulation with TNF-α induced the *p*12-LOX expression both in JB6 P+ and P– cells (Figure 4B), thus suggesting that *p*12-LOX is induced by inflammatory responses through the TNF-α/NF-κB pathway.

*Suppression of cell proliferation by inhibition of p12-LOX*

It has been shown that treatment with baicalein at 20 to 50 μM induces apoptosis and suppresses proliferation in several types of cancer cells (13,15-17). Therefore, the MTT assay was used to determine whether the proliferation of JB6 P+ cells is suppressed by baicalein at 5 μM, the same concentration used in this study. When $10^3$ cells were plated in each well of a 96-well microplate, JB6 P+ cells showed approximately 20% decreased proliferation compared with that of the control cells both at day 4 and day 6 (Figure 5). Treatment with NDGA at 5 μM also yielded a similar suppression of proliferation.

Importantly, however, the proliferation of JB6 P+ cells was suppressed more significantly by baicalein or NDGA when $10^2$ cells were plated in each well of a 96-well microplate (Figure 5). The number of drug-treated cells decreased by 90% of that of control cells at 6 days; the cells appeared to stop proliferation and they did not reach confluence after continuous culture for several days (data not shown). These results suggest that *p*12-LOX is therefore essential for the

proliferation of JB6 P+ cells when cells were plated at a low density. This is consistent with the results of the *in vivo* experiments (Figure 2C) showing that baicalein treatment did not affect cell proliferation in the established tumors, where the cell density was very high.

*Decrease of cloning efficiency of JB6 P+ cells by inhibition of p12-LOX*

These results prompted us to examine the possibility that *p*12-LOX is required for the proliferation from a solitary single cell. Such ability of proliferation from a single cell is one of the important factors that are required for tumorigenesis. The cloning efficiency of TPA-untreated or TPA-treated JB6 P+ cells were 54% and 44%, respectively (Figure 6B), indicating that the cloning efficiency was independent from TPA treatment. Notably, the transfection of siRNA for *p*12-LOX suppressed the cloning efficiency significantly to 41% and 31% in the TPA-untreated and TPA-treated JB6 P+ cells, respectively (Figure 6A and B). More importantly, baicalein treatment dramatically reduced the cloning efficiency to 3% and 5% in the TPA-untreated and -treated cells, respectively. These results indicate that *p*12-LOX is essential for the clonal proliferation from a solitary JB6 P+ cell, which should be important for the tumor promotion process. Although the suppression of cloning efficiency was more effective after baicalein treatment than that after siRNA transfection, this difference may be attributable to the transfection efficiency of siRNA.

*p12-LOX independent clonal proliferation of malignant cancer cells*

To investigate whether *p*12-LOX is required for the tumor promotion step in other tissues, the expression of *p*12-LOX in gastric tumors of *K19-Wnt1/C2mE* mice (5) as well as intestinal polyps

of *Apc*$^{\Delta 716}$ mice was examined (26). Interestingly, the *p*12-LOX expression was significantly induced in both gastric and intestinal tumor tissues (Figure 6C), suggesting the role of *p*12-LOX in tumorigenesis also in the gastrointestinal tract. Therefore, the cloning efficiency of gastric cancer cells (AGS and Kato-III) and colon cancer cells (DLD-1, SW480, and HCT-116) was examined. The treatment with baicalein at 5 μM significantly decreased the cloning efficiency of SW480 cells, although the colonies still formed in 44% of the plate wells (Figure 6D). The cloning efficiencies in other cell lines did not show significant decrease by the inhibition of *p*12-LOX. Accordingly, it is possible that progressing cancer cells acquire the ability for *p*12-LOX-independent proliferation from a solitary cell, unlike JB6 P+ cells.

**Discussion**

Accumulating evidence indicates that expression of *p*12-LOX is induced in several types of cancer tissues, whereas its expression is absent or weak in the normal tissues (6). Moreover, *p*12-LOX expression increases in the advanced stage of cancer tissues, and the *p*12-LOX pathway is involved in the suppression of apoptosis, induction of cell survival, and angiogenesis (10,15,19,21). These results suggest that the *p*12-LOX pathway plays a role in tumor development, progression and metastasis. In addition to these results, the present study demonstrated, by pharmacological and genetic experiments, that *p*12-LOX pathway is required for tumor promotion in mouse epidermal cell transformation. This is consistent with a previous report that disruption of the *p*12-LOX gene in mice suppresses the development of squamous cell carcinoma caused by an initiation/promotion protocol (23). Moreover, the present study has shown that treatment with 12(*S*)-HETE, a metabolite of *p*12-LOX, further increases the number of soft agar colonies in TPA-treated JB6 P+ cells. Accordingly, it is possible that formation of 12(*S*)-HETE functions as a rate-limiting factor for tumor promotion in epidermal cell transformation. This suggests that the induction level of *p*12-LOX may be positively correlated with the efficiency of tumor promotion.

It has been shown that baicalein, a *p*12-LOX, inhibitor induces apoptosis in several types of cancer cells (13,15-17). However, baicalein treatment at the concentration used in this study (5 μM) showed only mild suppressive effect on cell proliferation when $10^3$ JB6 P+ cells were plated in the microplate well. When the number of plating cells was reduced to $10^2$ cells, however, baicalein treatment at 5 μM significantly suppressed proliferation. Moreover, the cloning efficiency of JB6 P+ cells was suppressed dramatically by the same concentration of baicalein.

These results collectively indicate that 12(*S*)-HETE is required for the proliferation of small number of cells or a single cell. In the promotion step of tumorigenesis, the original "initiated" single cell or a small number of promoted cells needs to proliferate to form cluster of tumor cells. Accordingly, it is possible that the *p*12-LOX/12(*S*)-HETE pathway plays a key role in clonal proliferation of initiated cells during the promotion step. In contrast, the cloning efficiency of malignant gastric and colon cancer cells was not affected by inhibition of *p*12-LOX. It is possible that tumor cells derived from progressed cancer tissues have already acquired an ability of 12(*S*)-HETE-independent proliferation from a single cell.

These experiments showed that the expression of *p*12-LOX is induced in epidermal cells by stimulation with TNF-α, suggesting that an inflammatory host response induces *p*12-LOX expression through TNF-α/NF-κB pathway. It has been shown that TPA stimulation also activates NF-κB in JB6 P+ cells (31), which is required for transformation (29,30). It has been established that inflammation plays an important role in cancer development (32). Genetic studies have demonstrated that the activation of the TNF-α/NF-κB pathway is required for tumor development in the mouse colon and liver through the suppression of apoptosis in tumor cells and the induction of growth factors from stromal cells (33,34). Importantly, the expression of *p*12-LOX is induced not only in epidermal tumor cells but also in gastrointestinal tumors where an inflammatory response is associated (4,5). Accordingly, it is possible that *p*12-LOX is one of the tumor promoting factors induced by inflammatory responses through the TNF-α/NF-κB pathway in various types of tumors. It has been reported that AP-1 and NF-κB play a critical role in transformation of JB6 P+ cells, and activity of these factors is downregulated in JB6 P– cells (30,31). Our results indicate that the deficiency of *p*12-LOX in JB6 P– cells contributes to a

resistant phenotype to tumor promoter-induced transformation. Reactive oxygen species (ROS) also stimulate the neoplastic transformation of JB6 cells by activating AP-1 and NF-κB (31). Moreover, *p*12-LOX has also been reported to play a role in the ROS generation in neuroblastoma cells (35). Accordingly, it is possible that the inhibition of *p*12-LOX suppresses neoplastic transformation of JB6 P+ cells which is caused, at least in part, by the decrease of TPA-induced ROS generation.

These effects of the *p*12-LOX pathway on tumor promotion are similar to those of the COX-2/$PGE_2$ pathway in the gastrointestinal tumorigenesis. The activation of Wnt/β-catenin signaling causes the development of intestinal microadenoma (26). The subsequent induction of the COX-2/$PGE_2$ pathway in the microenvironment around the microadenoma is required for proliferation of small number of adenoma cells (3,36). Specifically, the inhibition of COX-2 or $PGE_2$ signaling suppresses proliferation of tumor cells in microadenoma (36,37). Importantly, these gastrointestinal tumors in mouse models also show the induction of the *p*12-LOX pathway as indicated in the present study. Therefore, it is possible that *p*12-LOX and COX-2 are two important pathways for tumor cell proliferation in the early stages. This is consistent with the findings of recent reports in which the polymorphism of either of COX-2 or *p*12-LOX was shown to be associated with an increased risk of colon cancer development (38). Moreover, both COX-2 expression and 12(*S*)-HETE synthesis are induced in mouse prostate tumors (39). On the other hand, baicalein treatment failed to induce a regression of the established tumors in the immunodeficient mice. Accordingly, it is conceivable that the *p*12-LOX/12(*S*)-HETE pathway plays an important role particularly in the early promotion step like COX-2/$PGE_2$ pathway, rather than in the progression stage.

In conclusion, the present results demonstrated that *p*12-LOX is required for tumor promotion during epidermal cell transformation, and 12(*S*)-HETE can accelerate tumorigenesis through enhancement of cloning efficiency. The expression of *p*12-LOX is induced also in the gastrointestinal tumors, suggesting that the *p*12-LOX pathway is an effective target for chemoprevention against skin carcinogenesis as well as gastrointestinal cancer.

**Acknowledgements**

We thank Manami Watanabe for valuable technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science.

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**Figure Legends**

**Figure 1.** The inhibition of *p*12-LOX in transformation-sensitive JB6 P+ cells suppresses colony formation in soft agar. (**A**) Representative RT-PCR results for the *p*12-LOX, epidermal-type 12-LOX (*e*12-LOX), leukocyte-type 12-LOX (*l*12-LOX), 5-LOX, 8-LOX are shown. The expression of *p*12-LOX is elevated in transformation-sensitive JB6 P+ cells, whereas it remains at the basal level in transformation-resistant JB6 P– cells. The expression of other LOX members is at the same level between JB6 P+ and JB6 P– cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. JB6 P+ and P– cells were stimulated with TPA (10 ng/ml) for 24 hours, and the mRNA levels were examined by RT-PCR chronologically at the respective time points indicated. Relative band intensity of *p*12-LOX to the level at 0 h is indicated. (**B**) Representative photographs of soft agar cultures of JB6 P+ (*top*) and JB6 P– (*bottom*) cells are shown (Giemsa staining). TPA-treatment (10 ng/ml) induced soft agar colony formation in JB6 P+ cells (*top, second panel from left*) but not in JB6 P– cells (*bottom, second panel from left*). Cells were treated with NDGA (5 μM), baicalein (5 μM), or AA-861 (10 μM) in the presence of TPA (*right 3 panels*). Note that treatment with a non-selective LOX inhibitor, NDGA and a selective *p*12-LOX inhibitor, baicalein resulted in significant suppression of soft agar colony formation, whereas the 5-LOX inhibitor, AA-861 treatment did not. (**C**) The mean number of colonies per field (mean ± S.D.) is shown. Treatments of the cells (drug treatment or siRNA transfection) are indicated below the histogram. *, $P < 0.05$ versus no-drug control of TPA-stimulated JB6 P+ cells. (**D**) Representative RT-PCR for *p*12-LOX in the siRNA-transfected JB6 P+ cells is shown. GAPDH was used as an internal control. (E) Immunoblotting for *p*12-LOX in the siRNA-transfected JB6 P+ cells is shown. β-Actin was used

as an internal control.

**Figure 2.** The inhibition of *p*12-LOX does not affect the tumor growth of JB6 P+ cells *in vivo*. (**A**) Photographs of TPA-stimulated JB6 P+ tumors developed in nude mice before (*left*) and after (*right*) baicalein treatment (20 mg/kg/day) at 26 and 40 days after the inoculation of tumor cells, respectively. (**B**) The tumor volume changes from day 25 to day 40 (mean ± S.E.) is shown. Solid lines indicate baicalein-treated mouse tumors, while dashed lines show no-drug control mouse tumors. (**C**) Representative photograph of BrdU incorporation (1 h) in the baicalein-treated tumor (*bottom*) and no-drug control tumor (*top*). *Bars* indicate 100 μm. Note that BrdU incorporation has increased in both baicalein treated- and untreated-mouse tumors. (**D**) Tumor volume at 1 week after palpable tumor developed in the baicalein-treated mice (from day 7) and no-treated mice (mean ± S.E.) is shown.

**Figure 3.** Treatment with 12(*S*)-HETE enhances TPA-induced neoplastic transformation of JB6 P+ cells. (**A**) Representative photographs of soft agar cultures are shown (Giemsa staining). TPA-negative control cells (*left*), TPA-stimulated and baicalein-treated cells (*center*), and TPA-stimulated but baicalein-untreated cells (*right*). The cells were pre-treated with 12(*S*)-HETE at 0.1 μM (*middle panels*) and 1 μM (*bottom panels*). Note that treatment with 12(*S*)-HETE restored soft agar colony formation of baicalein-treated JB6 P+ cells (*center panels*). The number of colonies increased by 12(*S*)-HETE even in the baicalein-untreated JB6 P+ cells (*right panels*). (**B**) The mean number of colonies per field is shown (mean ± S.D.). Treatments of the cells are indicated below the histogram. *, $P < 0.05$ versus 12(*S*)-HETE-untreated control of

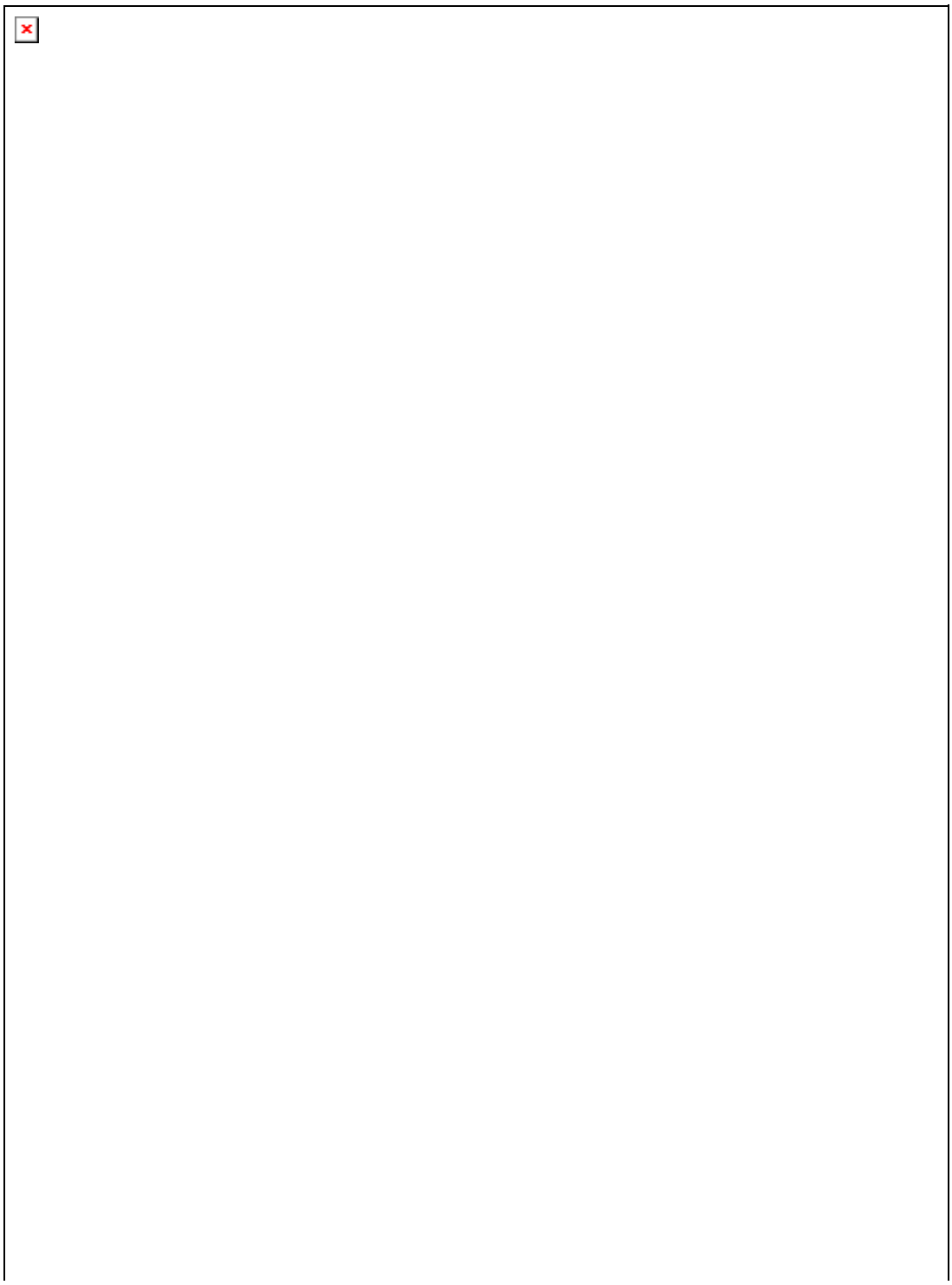
each group.

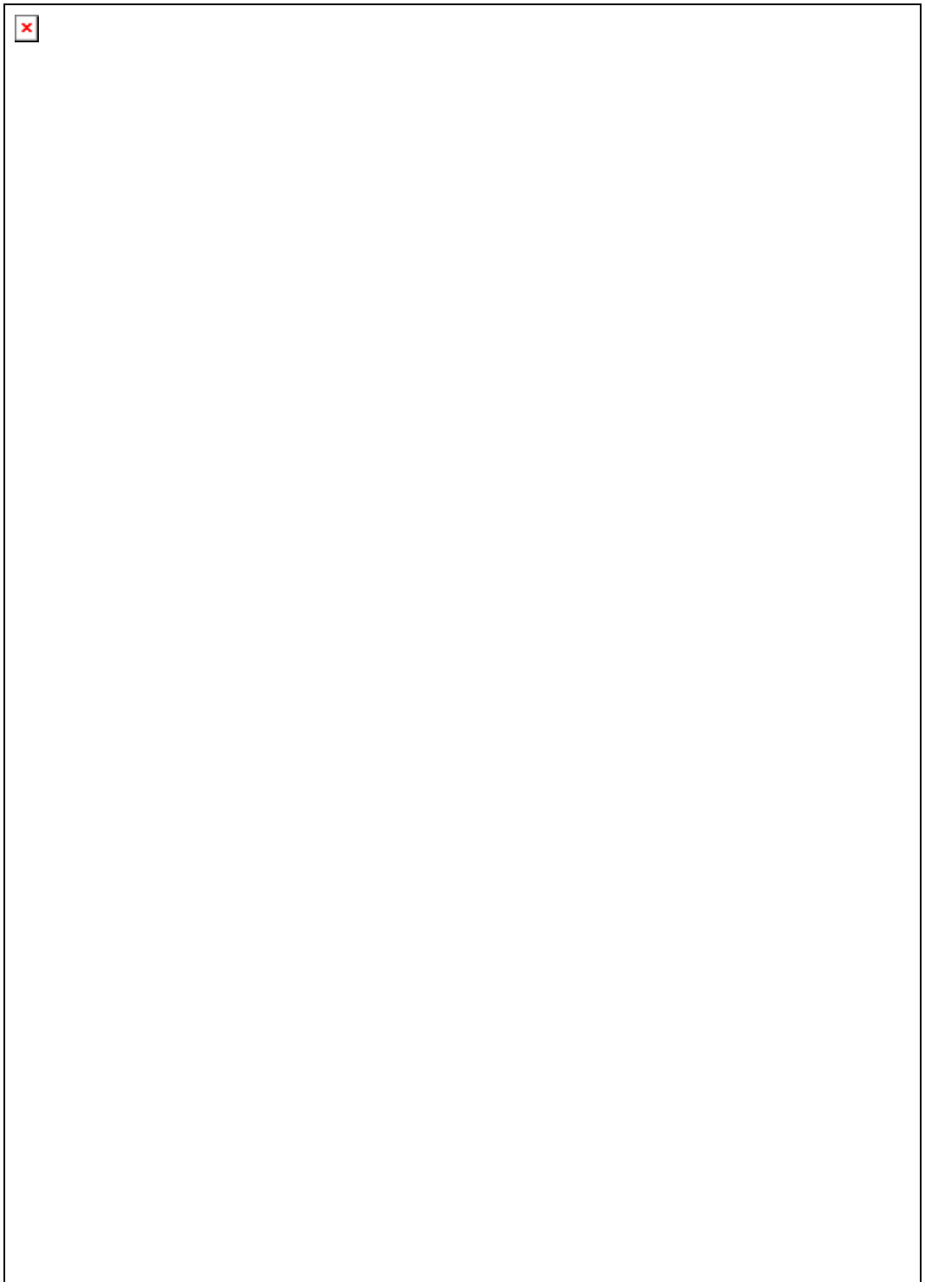
**Figure 4**. *p*12-LOX pathway is not required for TNF-α induced NF-κB activation, but is induced by TNF-α stimulation. (**A**) Relative NF-κB activity to the untreated JB6 P– level is shown (mean ± S.D.). Treatments of the cells are indicated below the histogram. *, $P < 0.05$ versus TNF-α-untreated level of JB6 P– and JB6 P+ cells, respectively. †, $P < 0.05$. (**B**) Representative RT-PCR results for *p*12-LOX in the TNF-α-simulated JB6 cells are shown. The expression of *p*12-LOX is induced both in JB6 P+ and P– cells by TNF-α stimulation. GAPDH was used as internal control.

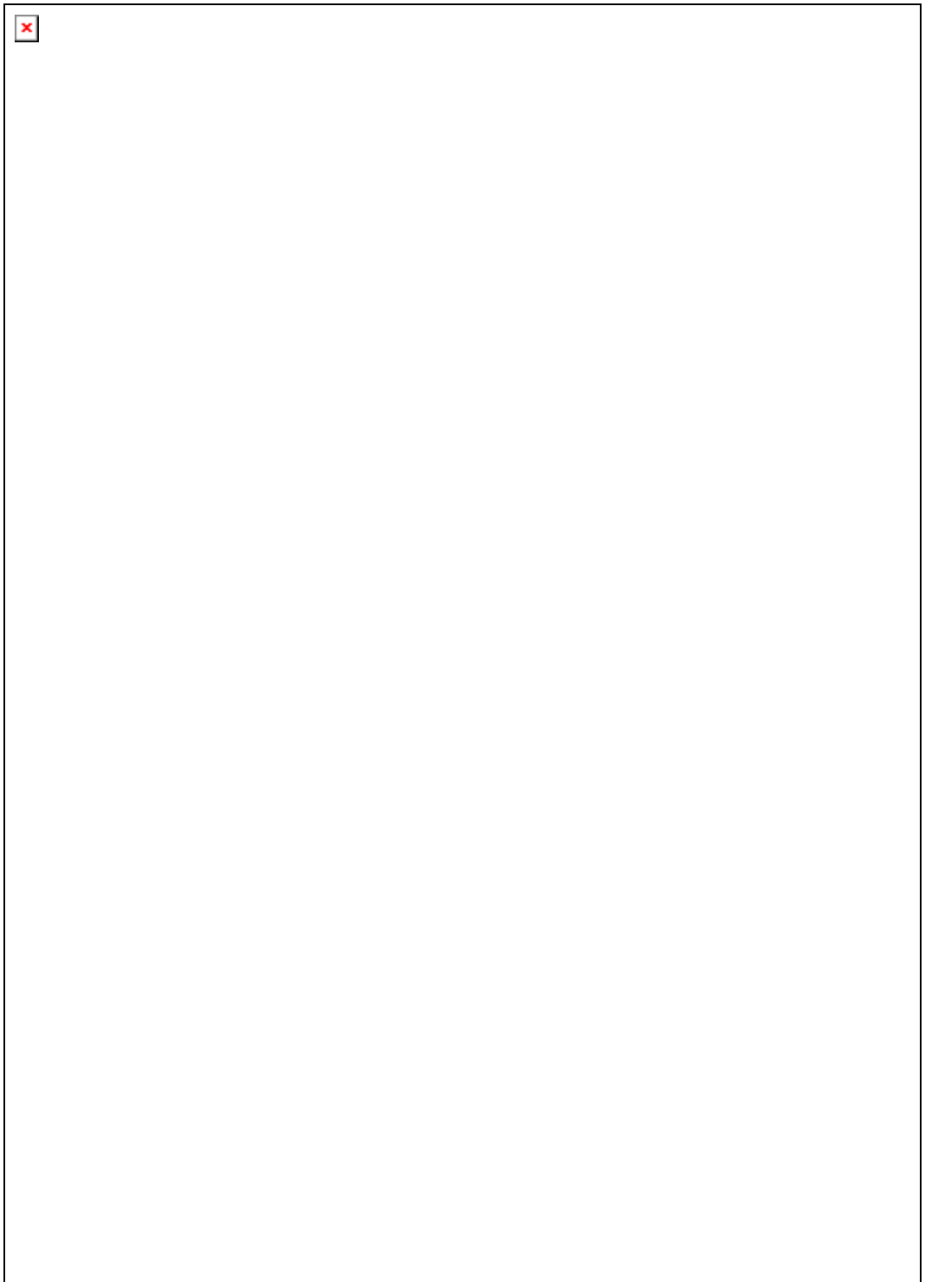
**Figure 5.** Inhibition of *p*12-LOX significantly suppresses JB6 P+ cell proliferation when cells are plated at a low density. Relative cell densities to no-drug control at day 4 (*left*) and day 6 (*right*) after plating are shown (MTT assay, mean ± S.D.). Open bars indicate relative cell density in the wells plated with $10^3$ cells, whereas black bars indicate $10^2$ cells. Note that treatment with NDGA (5 μM) and baicalein (5 μM) suppressed proliferation significantly in the wells plated with $10^2$ cells compared to that with $10^3$ cells. *, $P < 0.05$ versus no-drug control of the same cell-number group; and †, $P < 0.05$ versus $10^3$ cell-plated well of the same drug group.

**Figure 6.** Inhibition of *p*12-LOX suppresses the cloning efficiency of JB6 P+ cells. (**A**) Representative photographs of a microplate at 14 days after plating with no drug control, siRNA transfected, and baicalein treated cells (*left* to *right*), in which a single cell was seeded in each well (Giemsa staining). The wells containing proliferating cells are indicated with a circle. Note

that treatment with baicalein (5 μM) dramatically suppressed the cloning efficiency. (**B**) The cloning efficiency of JB6 P+ cells is indicated as histogram (mean ± S.D.). Treatments of the cells are indicated below the histogram. *, $P < 0.05$ versus baicalein-untreated control of each group. (**C**) Expression of *p*12-LOX is induced in gastric and intestinal tumors developed in *K19-Wnt1/C2mE* mice and $Apc^{\Delta 716}$ mice, respectively. Representative RT-PCR results are shown. *T* indicates tumor, while *N* indicates adjacent normal tissue. GAPDH was used as internal control. (**D**) The cloning efficiencies of gastric and colon cancer cells are indicated as histogram (mean ± S.D.). Treatments of the cells are indicated below the histogram. *, $P < 0.05$ versus baicalein-untreated control of each group.







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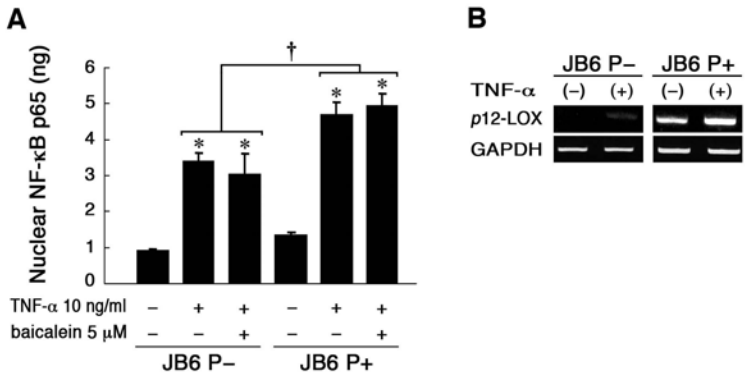

Fig. 5, Piao *et al.*