Review Article

# Roles of prostaglandins in facilitation of angiogenesis *in vivo*

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Angiogenesis, the formation of new blood vessels from the pre-existent microvasculature, is an essential component of wound repair and tumor growth. Nonsteroidal anti-inflammatories (NSAIDs) are known to suppress the incidence and progression of malignancies including colorectal cancers, and also to delay the wound healing. However, the precise mechanisms are not fully elucidated. Recent results obtained from prostaglandin (PG) receptor knockout mice indicate that host stromal PGE type receptor signaling is crucial in tumor-associated angiogenesis. Implanted tumor growth and tumor-associated angiogenesis were markedly suppressed in EP3 receptor knockout mice ($EP3^{-/-}$), in comparison with their wild-type counterparts (WT). Tumor-associated angiogenesis in WT depends on vascular endothelial growth factor (VEGF). Major VEGF-expressing cells in stroma were CD3/Mac-1 double-negative fibroblasts, and that stromal VEGF expression was markedly reduced in $EP3^{-/-}$. An EP3 receptor antagonist inhibited tumor growth and angiogenesis in WT. The wound healing process was significantly delayed in $EP3^{-/-}$. The bone marrow transplantation of $EP3^{-/-}$ bone marrow cells revealed that the recruitment of EP3-expressing bone marrow cells to the wound granulation tissues was critical to the healing of wounds. These demonstrate the significance of EP receptor signaling to the angiogenesis *in vivo*. Such signaling will be a good target for controlling angiogenesis in pathological conditions.

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## Process of Angiogenesis

We are complex multicellular organisms, and all cells in the body require a finely controlled supply of oxygen and neutrients[1-3]. The diffusion of oxygen in the tissues is limited to 100 to 200 $\mu$m, and a highly developed vascular system has evolved to ensure that all cells are within this distance of a supply of oxygen. Angiogenesis, a process of new blood vessel development from pre-existing vasculature is indispensable to maintain the integrity of the body. This involves endothelial cell division, selective degradation of the basement membrane and the surrounding extracellular matrix, endothelial cell migration, and the formation of a tubular structure. Once blood vessels have been established, the endothelial cells undergo tissue-specific changes to generate functionally active vessels. During embryogenesis, blood ves-

sels are formed by the differentiation of endothelial cell precursors (angioblasts), which associate to form primitive vessels. This process is called vasculogenesis[3,4].

Angiogenesis is subject to a complex control system with proangiogenic and antiangiogenic factors[3,4]. In adults, angiogenesis is tightly controlled by this "angiogenic balance", that is a physiological balance between the stimulatory and inhibitory signals for blood vessel formation. In normal status, the formation of new blood vessels occurs during wound healing, organ regeneration, and in the female reproductive system during ovulation, menstruation, and the formation of the placenta. It is also an important factor in several pathological processes such as tumor growth, rheumatoid arthritis, diabetic retinopathy, and psoriasis. A switch to the angiogenic phenotype depends on a local change in the balance between angiogenic stimulators and inhibitors[5].

Several growth factors, such as basic fibroblast growth factor, epidermal growth factor, and platelet derived growth factor, that regulate tube formation of endothelial cells and pericyte equipment are known. Besides these factors, the most important proangiogenic factors is vascular endothelial growth factor (VEGF). VEGF is a proangiogenic factor, that is identified first as a factor acting only on the endothelial cells. VEGF also potentiates microvascular hyperpermeability, which can both precede and accompany angiogenesis. The inflammatory mediators such as prostaglandins (PGs) are known to enhance the angiogenesis via up-regulation of VEGF. In this review, we summarize the properties and functions of PGs in the process of angiogenesis in malignancy and other conditions.

## Biosynthesis of prostaglandins (PGs)

The arachidonic acid (AA) cascade is the biosynthetic pathway which involves the release of the n-6 polyunsaturated fatty acid AA from the sn-2 position of membrane phospholipids by a phosholipase $A_2$ ($PLA_2$) enzyme, and its subsequent metabolism to bioactive prostaglandins (PGs), thromboxanes, leukotrienes, and epoxy fatty acids, by cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 epoxygenase enzymes, coupled to specific terminal synthases. AA and its end-products are involved in physiological processes and in pathological ones. While pharmacological inhibitors can be used to investigate the role of key enzymes involved in AA release and metabolism of AA in physiological and pathological models, the lack, in some cases, of specific inhibitors or of a complete pharmacological inhibition, and standardization of dosing paradigms complicate the studies. Two COX isoforms have been identified: COX-1 is constitutively expressed in various tissues, whereas COX-2 is induced by mitogens, cytokines, and tumor promoters[6]. COX regulates the formation of an unstable endoperoxide intermediate, $PGH_2$, which, in turn, is metabolized to $PGD_2$, $PGE_2$, $PGF_{2\alpha}$, $PGI_2$, and thromboxane (TX)$A_2$ by cell-specific isomerases and synthases. Prostanoids formed are immediately released outside of the cell. They are either chemically or metabolically unstable, thus prostanoids are believed to work only locally, near their site of production. $PGI_2$ and $TXA_2$ spontaneously degrade into inactive compounds under physiological conditions, and other PGs are enzymatically inactivated during a single passage through the lung. $PGD_2$ and $PGE_2$ are slowly dehydrated in biological fluids containing serum albumin to be the cyclopentenone PGs.

## Receptors for PGs

Prostanoids exert their actions via membrane receptors on the surface of target cells. Genes for each of these receptors have been disrupted and the corresponding knockout mice have been produced[7]. Furthermore, with the use of the cloned receptors, agonists and antagonists highly selective for each of the four EP subtypes have been developed. Eight types and subtypes of membrane prostanoid receptors are conserved in mammals from mouse to human[7]: the PGD receptor (DP), four subtypes of the PGE receptor (EP1, EP2, EP3, and EP4), the PGF receptor (FP), the PGI receptor (IP), and the TXA receptor (TP). All receptors are G protein-coupled rhodopsin-type receptors with seven transmembrane domains, and each is encoded by different genes. There are several splice variants in the EP3, FP, and TP receptors, which differ only in their C-terminal tails. Among the eight types and subtypes, the IP, DP, EP2, and EP4 receptors mediate a cAMP rise, whereas the TP, FP, and EP1 receptors induce calcium mobilization. EP3 has several splice variants, which increases or decreases cAMP levels, and induce calcium mobilization. The effects of prostanoids on these G protein-coupled signaling pathways are reported to be changed in a ligand concentration-dependent manner. $PGI_2$ analogs bind to IP and activate adenylate cyclase via Gs in a dose-dependent manner, but higher concentrations of the ligand couple to IP and activate phospholipase C to mobilize calcium ions via Gq. There are four subtypes of the receptor for $PGE_2$ although the other prostanoids each have only a single receptor. The homology of amino acid sequences between different types of the receptors within each functional group is much higher than that found among the four PGE receptor subtypes. The phylogenetic tree derived from receptor homologies indicates that prostanoid receptors originated from the primitive PGE receptor. Other PGs and TX receptors

Table 1 Roles of prostaglandins revealed by studies using prostaglandin receptor knockout mice

| PGs | Receptors | Pathophysiological roles |
|---|---|---|
| $PGD_2$ | DP | A mediator of allergic asthma |
| $PGE_2$ | EP1 | inhibition of gastric motor activity |
| | EP2 | Ovulation and fertilization, Salt-sensitive hypertension |
| | EP3 | A mediator of febrile response, Acid-induced bicarbonate secretion in duodenum, Urinary concentration |
| | EP4 | Closure of ductus arteriosus, Bone resorption |
| $PGI_2$ | IP | Antithrombotic function, Adaptive cytoprotection of stomach, Acetic acid-induced writhing reaction |
| $PGF_{2\alpha}$ | FP | An essential inducer of labor |
| $TXA_2$ | TP | Hemostasis, Endotoxicin-induced hepatic microcirculatory dysfunction |

subsequently evolved from functionally related PGE receptor subtypes by gene duplication. The roles of PGs in various physiological and pathophysiological processes have been clarified with mice deficient in each prostanoid receptor. The findings including ours with use of knockout mice are summarized in Table 1. In this review article, we will discuss the significance of the findings related to angiogenesis *in vivo*.

## Roles of PGs in angiogenesis indispensable to wound healing

Angiogenesis is believed to be an essential component of normal wound repair. Immediately following injury, it delivers oxygen, nutrients and inflammatory cells to the site of injury. It also assists in the development of granulation tissue formation and ultimately wound closure. Both angiogenic agonists and antagonists are identified at various stages of the wound repair process[8], suggesting a dynamic balance of stimulators and inhibitors that favor either vascular growth or regression[9]. It has been previously reported that E type PGs have a proangiogenic activity in corneal tests[10] and in the chorioallantoic membrane (CAM) technique[11]. Further, Form and Auerbach reported that $PGE_2$ strongly induced angiogenesis on the CAM of 8-day-old chicken embryos, but $PGA_2$, $PGF_2$, and a derivative of $TXA_2$ did not. A report[12] described that the endothelial migration was mediated by COX-2. This experiment was performed *in vitro* using confluent monolayer endothelial cells stimulated with PMA, and the authors also reported that corneal angiogenesis was suppressed with COX-2 inhibitor, suggesting the involvement of COX-2 products *in vivo*. These suggested that the endogenous PGs regulated angiogenesis not only in physiological conditions but also in pathological ones.

The roles of COX-2 derived PGs in wound healing and the PG receptor signaling relevant to wound-induced angiogenesis were reported recently[13]. When full-thickness skin wounds were created in mice, and angiogenesis in wound granulation tissues was estimated, wound closure and reepithelization was delayed in mice treated with COX-2 inhibitors. The wound closure and reepithelization in EP3 receptor knockout mice ($EP3^{-/-}$) were significantly delayed compared with their wild type mice (WT) (Fig. 1), whereas those in $EP1^{-/-}$, $EP2^{-/-}$, and $EP4^{-/-}$ were not delayed. Wound-induced angiogenesis in $EP3^{-/-}$ was significantly inhibited compared with that in WT (Fig. 2). Reduced wound-induced angiogenesis in $EP3^{-/-}$ was accompanied with poor development of granulation tissues under the wound. VEGF expression in wound granulation tissues in $EP3^{-/-}$ was markedly less than that in WT. Wound closure in WT was delayed significantly by VEGF neutralizing antibody compared with control IgG. Wound-induced angiogenesis and wound closure were significantly suppressed in $EP3^{-/-}$ bone marrow transplantation mice, compared with those in WT bone marrow transplantation mice. These were accompanied with the reductions in accumulation of VEGF-expressing cells in wound granulation tissues and in recruitment of VEGF receptor 1-expressing leukocytes in peripheral circulation. These results indicated that the recruitment of EP3-expressing cells to wound granulation tissues is critical for surgical wound healing and angiogenesis via up-regulation of VEGF[13].

## Roles of PGs in tumor-associated angiogenesis

Several epidemiological studies revealed a 40-50% reduction in mortality from colorectal cancer in individuals taking nonste-

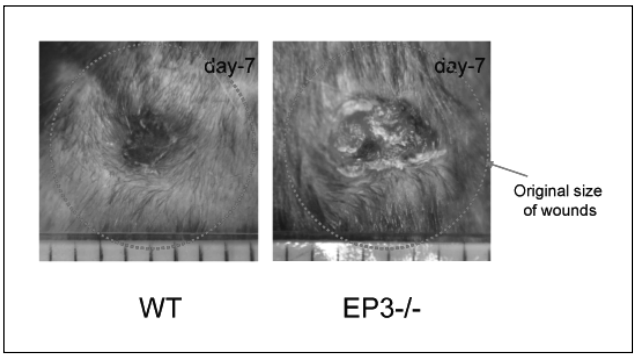


Fig.1 Delay in wound healing in EP3 knockout mice
Tipical appearance of wounds 7 days after wounding.
Surgical wounds were made on the backs of EP3 receptor knockout mice (EP3$^{-/-}$) and of their wild-type counterparts (WT).
The original diameter of the wounds was 8 mm.
One division on the scale below the wound represents 1 mm.
Cited from reference 13 with permission.

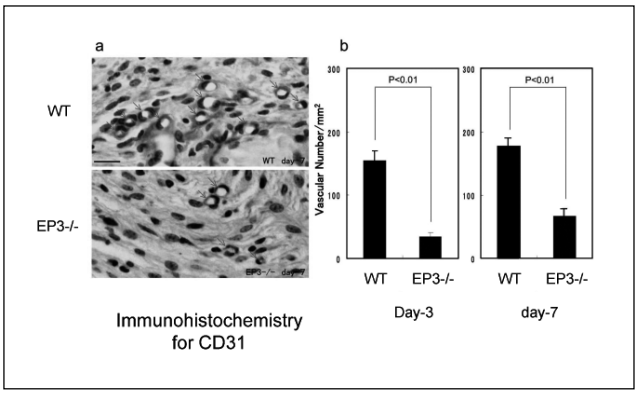


Fig.2 Reduced angiogenesis in wound granulation tissues in EP3 knockout mice
a; Immunohistochemical localization of CD31 in wound granulation tissues isolated from mice 7 days after wounding.
b; Vasculare density in wound granulation tissues isolated from mice 3 and 7 days after wounding.
Cited from reference 13 with permission.

roidal anti-inflammatory drugs (NSAIDs), and other evidence suggests that they also affect the incidence and progression of other types of cancer, pointing to a possible role of COX in tumor formation[14]. NSAIDs that inhibit COXs and suppress PG biosynthesis have been widely used as anti-inflammatory, antipyretic and analgesic agents. Disruption of the COX-2 gene in mice reduced the number and size of intestinal polyps generated by a mutation in the adenomatous polyposis (APC) gene, thus verifying a role for COX-2 in the generation of colon tumors[15]. COX-2 selective inhibitors were expected to act as a "super aspirin" which would not exhibit the adverse effects typical of classical NSAIDs[6]. However, it has been reported that some organs, such as the kidney, expressed COX-2 constitutively[6], and that COX-2 was necessary for the kidney to mature after birth[16]. Thus, selective PG receptor signaling inhibition may be a more effective form of treatment of patients than COX-2 inhibition. A wide range of mechanisms of the anti-tumor actions of NSAIDs, some of which are unrelated to inhibition of cyclooxygenase activity, and of subsequent PG formation has been proposed[17-19].

Evidence that PG receptor signaling is relevant to tumor development has been accumulated through the use of prostaglandin receptor knockout mice (Table 2). We must emphasize here that the models must be carefully selected according to the interests of the researchers. Analysis of a tumor implantation model in some knockout mice is suitable for observing the host stromal responses that facilitate tumorigenesis, since the lack of the receptors is observable in the host stroma, although tumor cells may, or may not, express the receptors in question, depending on the cell lines implanted. In tumor implantation experiment, differences of phenotype such as tumor growth and tumor-associated angiogenesis in the mice are highly dependent on the receptor signaling in the host. The models of other categories are developed to see the effect of PG receptor signaling on tumor cells themselves (tumor cell-autonomous effect), as induced by the mutation of tumor epithelial cells in addition to the host stromal effect. The most successful example of the latter is the marked reduction in polyp number in *Apc716* mice (a model of FAP) against a Cox-2$^{-/-}$ background, in comparison with control animals[15]. In Cox-2$^{-/-}$ background mice, COX-2 was deficient both in polyp epithelial cells and in stromal cells.

It was previously reported that $PGE_2$ can promote colorectal cancer growth, in part through the activation of the $PGE_2$ receptor subtype EP1 and EP4. The experiments using knockout mice with a colon carcinogen, azoxymethane, revealed the significant suppression of aberrant crypt foci in EP1-deficient mice together with EP4-deficient mice. The suppression was limited in both cases, suggesting the possible involvement of other receptors or mechanisms. Moreover, aberrant crypt formation represents an initial step in carcinogenesis, and many events precede the development of colon cancer. It is likely that PGs are also involved in other steps and mechanisms. The expression of COX-2, but not COX-1, is elevated in many colorectal cancers, and the protein has been localized to both stromal and epithelial compartments. One mechanism by which elevated COX-2 promotes car-

Table 2 Prostaglandin $E_2$ and $I_2$ receptor signaling and relationship with cancer development

| Prostanoid | Receptor | G protein | Signaling pathway | Relationship with cancer development | Refs |
|---|---|---|---|---|---|
| $PGE_2$ | EP1 | Gs | PI response | EP1 antagonist decreases incidence of aberrant crypt foci in azoxymethane-treated rats or *APC*$^{Min}$ mice. | 44) |
| | | | | EP1-null mice are partly resistant to azoxymethane-induced aberrant crypt foci. | 32) |
| | EP2 | Gs | cAMP increase | In EP2-null mice, the number of *APC*$^{\Delta 716}$ intestinal polyps and the intensity of angiogenesis and VEGF expression are decreased. | 23,24) |
| | | | | EP2-null mice exhibit cancer-associated immunodeficiency and dendritic cell abnormalities, but have no effects on tumor-associated angiogenesis and VEGE induction. | 45) |
| | EP3 | Gs | cAMP increase | EP-3-null mice exhibit reduced tumor-associated angiogenesis and tumor growth through the induction of VEGF. | 20) |
| | | Gi | cAMP decrease | — | |
| | | Gq | PI response | — | |
| | EP4 | Gs | cAMP increase | EP4-null mice decrease tumor cell motility. | 46) |
| $PGI_2$ | IP | Gs | cAMP increase | Tumor metastasis inhibition with prostacyclin analogue. | 47) |
| | | Gq | PI response | — | |

PG, prostaglandin; VEGF, vascular endothelial growth factor

cinogenesis is through stimulation of tumor-associated angiogenesis.

In tumor implantation models, the involvement of $PGE_2$-EP receptor signaling in tumor-induced angiogenesis was tested[20]. It was reported that in the four subtypes of EP receptor knockout mice, in $IP^{-/-}$, and in their WT counterparts, tumor-associated angiogenesis in $EP3^{-/-}$ mice was suppressed by nearly 80%, although partial reduction of angiogenesis was observed in $EP2^{-/-}$ mice[20]. Histological examination of tumors formed in $EP3^{-/-}$ mice revealed a low level of vascularization and distinct boundaries with the surrounding normal tissue. In spite of the implantation of the same number of tumor cells, differences of tumor growth and tumor-associated angiogenesis were observed in these mice, strongly suggesting that the EP3 receptor in the host, not on the tumor cells, has a major, indeed, the critical role in tumor-induced angiogenesis and tumor growth. Staining for COX-2 was apparent in tumors together with the stromal cells and endothelial cells[20]. In WT mice, VEGF was abundant in the surrounding stromal cells, whose major components were Mac-1 and CD3 negative fibroblast-like cells. Expression of VEGF was markedly reduced in $EP3^{-/-}$ mice. Gel shift assays revealed that AP-1 may be important in VEGF expression and angiogenesis[20], although other factors, such as HIF-1, whose activation was related to EP1, EP2, and EP4 signaling[21,22], were not ruled out.

It was reported that angiogenesis and growth of polyps were EP2-dependent when the APC gene was mutated[23,24]. The reports of those authors stated that the major elements that express

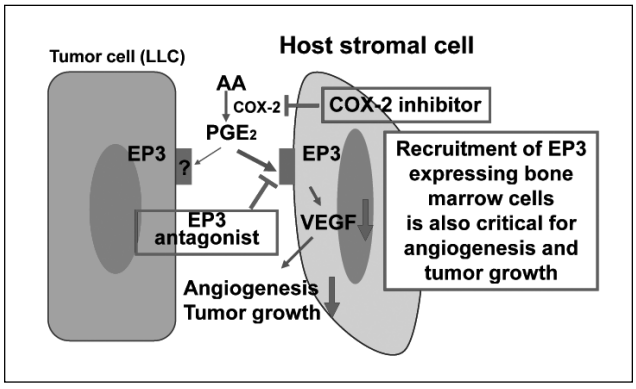


Fig.3 Targets for controlling tumor-associated angiogenesis regulated by endogenous prostaglandins

Stromal EP3 signaling is a key regulator of tumor angiogenesis and tumor growth.

COX-2 inihibition and EP3 blockade are effective in preventing tumor growth and angiogenesis.

Controlling host stromal function by modification of inflammatory signaling relevant to tumor angiogenesis may also become a useful strategy to treat the solid tumors such as cancers.

COX-2 are stromal cells around the intestinal polyps. The tumor stromal cells which produced VEGF so as to facilitate angiogenesis and tumor growth were CD3 and Mac-1 double negative fibroblasts[20]. Fibroblasts exhibit heterogeneity in various biological factors including prostaglandin generating systems and

receptor systems[25-27]. The authors of the above report[24] did not show the microvessel density in EP3$^{-/-}$ with APC mutation, and the reduction percentage of angiogenesis in APC-mutated EP2$^{-/-}$ mice was approximately 30% at best. The major EP receptor expressed in the subcutaneous tissues of WT mice was EP3[20], which was not expressed in the intestine[23]. These findings suggested that tumor-associated angiogenesis may be regulated in a site-specific fashion, and may be related to the heterogeneity of the stromal fibroblasts. It was reported that EP2-null mice bearing subcutaneous tumor cells exhibit cancer-associated immunodeficiency and dendritic cell abnormalities, but surprisingly have no effects on tumor-associated angiogenesis and VEGF induction, in spite of a partial reduction in tumor growth. This indicates that intracellular signaling linked to EP2 receptor activation also may be heterogenous.

The host microenvironment is believed to influence tumor progression[28,29]. As mentioned above, PGs may be one of the important determinants of tumor-host communications. Examination of human colorectal cancer has revealed marked COX-2 expression not only in cancer cells but also in the stroma that surrounds them[30]. COX-2 knockout mice also exemplify the significance of stromal COX-2 in tumor-induced angiogenesis[31]. The study using EP3$^{-/-}$ mice revealed that COX-2-expressing stromal cells around the tumors, or the tumor cells themselves, or both, synthesize and release $PGE_2$ into the tumor microenvironment; and that $PGE_2$ then acts on the stromal cells that express EP3 receptor to induce proangiogenic factor production and consequent angiogenesis (Fig. 3). EP3 receptor signaling is important in angiogenesis promotion, but it cannot be ruled out that EP2 receptor signaling may facilitate angiogenesis synergistically.

## PGs as targets for controlling angiogenesis *in vivo*

As discussed, highly selective EP antagonists such as EP3 and EP2 receptor antagonists therefore exhibit beneficial actions on the stromal cells, and may be a good choice as novel therapeutic tools against cancer. Administration of an EP3 antagonist to the tumor-bearing mice significantly suppressed tumor-associated angiogenesis and tumor growth in WT mice[20]. By contrast, administration of neither an EP1 nor an EP4 antagonist, both previously developed[32,33], did so. Furthermore, such a preventive effect of an EP3 antagonist was not seen in EP3$^{-/-}$ mice[20], suggesting that EP3 receptors expressed on the tumor cells themselves did not exhibit any significant roles in tumor-associated angiogenesis or tumor growth, since the EP3 antagonist administered may have effectively blocked the EP3 receptors on the tumor cells (Fig. 3). These facts confirmed the result obtained in EP3$^{-/-}$ mice, namely, that EP3 receptor signaling acts predominantly on the host stroma. This signaling on the stromal cells was relevant to the induction of the a potent proangiogenic growth factor VEGF, and up-regulated VEGF certainly has a proangiogenic action, and facilitates tumor growth (Fig. 3)[20]. A highly selective EP3 antagonist therefore exhibits preventive action on the stromal cells, and is expected to become a novel therapeutic asset against cancer.

Inflammation is a local protective response to harmful stimuli. Recent results have expanded the concept that inflammation is an important factor in facilitating tumor growth[34]. In fact, many cancers arise from the sites of infection, chronic irritation, and inflammation. PGs are the major proinflammatory mediators, and increase inflammation induced by other chemical mediators. The tumor stromal reaction can be characterized by the proliferation of tissues including fibroblasts, which can facilitate angiogenesis and probably lymphangiogenesis. The results obtained from the sponge implantation model can support the significance of the proliferation or infiltration, or both, of inflammatory cells in the site where angiogenesis occurred[35-40]. The stromal fibroblasts may be derived from the bone marrow[34], and from tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes[41]. Targeting tumor angiogenesis with exogenous genes to tumor angiogenesis was performed by transplantation of genetically modified hematopoietic stem cells[42]. Thus, transplantation of EP receptor null bone marrow cells may provide the means for targeted inhibition of tumor-associated angiogenesis. Control of the inflammatory responses in the tumor microenvironment where EP receptor expressing cells are accumulating is also likely to be a novel therapeutic approach against cancer, which now annually causes some 550,000 deaths in US[43].

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