Furthermore, FGF-4 is expressed in approximately one-third of primary human germ cell tumors. Through an immunohistochemical study, FGF-8, FGF-4, and FGFR1 are found to be expressed predominantly in nonseminomatous and highly proliferative testicular germ cell tumors (Suzuki et al., 2001). These observations indicate that FGF-4 is a potential target for the treatment of human testicular tumors. Minakuchi et al. reported that human FGF-4 small interfering RNA (siRNA) inhibited the cell growth of a human testicular tumor cell line (Minakuchi et al., 2004), which showed high levels of *FGF-4* mRNA expression in vitro and in vivo (Yoshida et al., 1988a). Data from this report show that FGF-4 siRNA transfer might be a significant novel method for inhibition of tumor growth in vivo.

Several reports showed the genomic amplification of *FGF-4* and *FGF-3* in a tumor. Kiuru-Kuhlefelt et al. reported that the DNA copy number and the expression of *FGF-4* were up-regulated in Kaposi's sarcoma (Kiuru-Kuhlefelt et al., 2000). Tsuda et al. showed that coamplification of the *FGF-4* and *FGF-3* genes was observed in esophageal carcinomas, primary tumor tissues, and metastatic tumors (Tsuda et al., 1989). The coamplification of the *FGF-4* and *FGF-3* genes had a tendency to correlate with the clinical stage. Theillet et al. reported that 1 of 13 melanomas (8%), 3 of 43 bladder tumors (7%), and 41 of 238 breast carcinomas (17%) contained amplified *FGF-4* and *FGF-3* (Theillet et al., 1989). Amplification of the chromosomal locus of the *FGF-4* and *FGF-3* genes might participate in carcinogenesis, in progression, and particularly in the metastasis of carcinomas. However, the role of FGF-4 in oncogenesis and metastasis still needs further investigation.

FUNCTION AND REGULATION OF FGF-4 IN EMBRYOGENESIS

Fgf-4 is expressed in preimplantation mouse blastocysts and is present in inner cell mass (ICM; Niswander and Martin, 1992; Rappolee et al., 1994). Mouse embryos expressed *Fgf-4* mRNA from the 1-cell stage. And *Fgf-4* mRNA is found as a maternal transcript, and is expressed at the blastocyst stage. In 1995, Feldman et al. showed an essential role of Fgf-4 in embryogenesis (Feldman et al., 1995). Inactivation of the *Fgf-4* gene in mice results in embryonic lethality after implantation. In contrast, proliferation of the ICM is rescued by addition of FGF-4 protein in *Fgf-4* null embryos. Drucker and Goldfarb (1993) also showed that the expression of murine *Fgf-4* was detected in primitive streak (E7.5–E8.5), paraxial presomitic mesoderm in the trunk, primitive neuroectoderm, pharyngeal pouch endoderm, branchial arch ectoderm, limb apical ectoderm, and skeletal myoblast groups. During the early stages of gastrulation, expression becomes restricted to the primitive streak (Niswander and Martin, 1992). Then the expression of *Fgf-4* is detected in the tail bud. Furthermore, *Fgf-4* mRNA is detected after the three primary germ layers are established and organogenesis begins. Of interest, FGF-4 can induce mesoderm formation in isolated *Xenopus laevis* animal pole explants and stimulate DNA synthesis in mammalian fibroblasts (Paterno et al., 1989). Embryonic FGF (eFGF), which is the amphibian orthologue of FGF-4, has mesoderm-inducing activity (Isaacs et al., 1992). *eFGF* mRNA is expressed maternally and zygotically with a peak during the gastrula stage. The zygotic expression is restricted in the posterior of the body axis and later in the tail bud. These results suggest that the FGF-4 has multiple roles during vertebrate embryogenesis.

Fgf-4 mRNA was detected in the Days 11 and 12 embryo, where it is localized to the apical ectodermal ridge (AER) of the limb bud (Suzuki et al., 1992). This structure is well known for its role in promoting distal outgrowth of the developing limb bud. The expression of *Fgf-4* mRNA is detected in both fore- and hindlimbs. FGF-4 stimulates proliferation of cells in the distal mesenchyme and maintains a signal from the posterior to the distal (Niswander et al., 1993; Niswander and Martin, 1993). Ochiya et al. established an organ culture system to allow mouse limb bud at 9.5–10 days postcoitus (dpc) embryo to differentiate into a limb at 12.5 dpc embryo (Ochiya et al., 1995). Exposure of embryonal limb bud explants to antisense oligodeoxynucleotides of *Fgf-4* blocks limb development. These results suggest that Fgf-4 plays the major function of the AER. However, Moon et al. showed *Fgf-4* conditional mutants have normal forelimbs (Moon et al., 2000). Furthermore, *Fgf-4* is not required for normal limb development or *Shh* expression in the zone of polarizing activity. Although *Fgf-4* expression is increased in the AER of *Fgf-8* conditional mutant forelimbs, *Shh* expression is decreased at E11.5 in *Fgf-8* conditional mutants (Moon and Capecchi, 2000). Inactivation of Fgf-8 in early limb ectoderm causes a reduction in limb-bud size, a delay in Shh expression, and misregulation of Fgf-4 expression (Lewandoski et al., 2000). Moreover, activation of *Fgf-4* in an *Fgf-8*-null limb bud causes polysyndactyly, but it rescues all the skeletal defects that result from loss of Fgf-8 function (Lu et al., 2006). These results indicate that FGF-4 and FGF-8 coordinately contribute to limb development. However, Fgf-8, rather than Fgf-4, is required to maintain *Shh* expression in the AER. Sun et al. suggest the hypothesis that there is a positive feedback mechanism between SHH from the ZPA and FGF-4 from the AER (Sun et al., 2000).

In mouse development, FGF-4 inhibits apoptosis in the dental mesenchyme when applied locally (Vaahtokari et al., 1996). Lymphoid enhancer factor (LEF1), a nuclear mediator of Wnt signaling, is a critical epithelial survival factor during tooth morphogenesis. The *Fgf-4* gene acts as a direct transcriptional target for LEF1 and shows that FGF-4 can rescue the developmental arrest of Lef1 (−/−) tooth germs (Fig. 1; Kratochwil et al., 2002). Taken together, these data indicate that FGF-4 can account for the function of LEF1 in tooth development, allowing for a communication between epithelium and mesenchyme.

FGF-2, FGF-1, and FGF-4 are exclusively detected in the endoderm at stage 5 and later in the myocardium of chick embryo, appearing as punctate cytoplasmic deposits (Zhu et al., 1996). Expression of all FGFs peaks at stages 18–24, decreasing thereafter in parallel with reduced myocardial cell proliferation. FGF-4 supports the proliferation and differentiation of precardiac myoblasts in vitro. Sugi et al.

reported that FGF-4 was expressed in cushion mesenchymal cells in the chick (Sugi et al., 2003). Addition of FGF-4 induces proliferation of cushion mesenchymal in vitro and in vivo. These findings support the hypothesis that FGF-4 is involved in the regulation of early heart development through paracrine and autocrine mechanisms.

FGF-4-mediated signaling is needed for establishing gut tube domains in chick embryos (Dessimoz et al., 2006). It is expressed in gastrulation and somite stage embryos in the close of the posterior endoderm. Exposure of the endoderm to FGF-4 at the gastrula stage reveals that it promotes a posterior gut cell fate and represses anterior endoderm cell fate. Additionally, FGF-4 represses the anterior endoderm markers and inhibits foregut morphogenesis. Disruption of FGF signaling demonstrates that FGF signaling is necessary for establishing midgut gene expression.

Fraidenraich et al. showed that a 3′-untranslated region enhancer region of *Fgf-4* gene is a target for the myogenic bHLH transcription factors MYF5 and MYOD in the myotomes and AER (Fraidenraich et al., 2000). Furthermore, Iwahori et al. found a minimal *Fgf-4* enhancer, which is a binding site for the GATA family of transcription factors in the myotomes (Iwahori et al., 2004). Fisher et al. showed regulation of the myogenic regulatory factor XmyoD in the skeletal muscle lineage of *Xenopus laevis*. The signalling molecule eFGF can directly induce the expression of XmyoD in myogenic cells (Fisher et al., 2002).

Epithelial–mesenchymal transitions (EMT) are important for morphogenesis during embryonic development (Thiery, 2002). FGFR1 promotes the EMT and morphogenesis of mesoderm at the primitive streak by controlling Snail and E-cadherin expression (Ciruna and Rossant, 2001). Snail and Twist are key inducers of EMT that represses E-cadherin expression (Batlle et al., 2000; Cano et al., 2000). Furthermore, Twist and Snail contribute to metastasis by promoting an EMT (Cano et al., 2000; Yang et al., 2004). During vertebrate gastrulation, EMT is necessary for migration of mesoderm from the primitive streak. To regulate mesoderm migration, Fgf signaling is required for expression of Snail (Zohn et al., 2006). Exogenous FGF-4 in tooth and FGF-2 in palate induce the expression of Twist and Snail in isolated mesenchymal explants (Rice et al., 2005). Moreover, in the Twist (−/−) forelimb bud, *Fgf-4* is not expressed (O'Rourke et al., 2002). β-Catenin has been shown to activate LEF-1 transcription during EMT induced in vitro by c-Fos. LEF-1 can induce EMT directly when its transcription activity is activated by stable nuclear β-catenin (Kim et al., 2002). These data suggest novel insights into the molecular basis and requirement of FGF-4 in the process of EMT.

Recently, Sweetman et al. found a restricted expression of microRNAs (miRNAs) in developing somites, in particular the developing myotome (Sweetman et al., 2006). The miRNAs are recently discovered short, noncoding RNAs, which regulate gene expression in metazoans. FGF-4–mediated signaling negatively regulates the initiation of miRNA expression during somite development. From this view, miRNAs is a potential regulator of the FGF-4 mediated developmental mechanism. As stated above, FGF-4 has a multiple function in vertebrate development. This newly discovered molecular mechanism involving miRNAs spotlights the complicated dynamics in development. The importance of FGF-4 in embryogenesis has been shown in many organisms. Further investigations to clarify the complex molecular mechanisms for FGF-4 will provide the answer for its function in stem cell and oncogenesis.

ROLE OF FGF-4 IN EMBRYONIC STEM CELLS AND TROPHOBLAST STEM CELLS

Expression of FGF-4 is restricted to undifferentiated embryonic stem (ES) cells and embryonal carcinoma (EC) cell lines such as F9 and P19, but not in differentiated cells (Yoshida et al., 1988b; Velcich et al. 1989; Hebert et al., 1990; Schoorlemmer and Kruijer, 1991). Stem cell specific expression of FGF-4 is controlled by a distally localized enhancer. This enhancer contains a consensus octamer binding site that controls positive regulation in EC and ES cells. The Sox2/Oct-3/4 complex, which is vital for normal pluripotent cell development and maintenance, can bind on *FGF-4* enhancer DNA sequences and promote transcriptional activation of FGF-4 (Fig. 2; Yuan et al., 1995). Oct-3/4 mRNA is expressed in both human and mouse oocytes and blastocysts (Schöler et al., 1989; Curatola and Basilico, 1990; Schöler, 1991). In the preimplantation stages of mouse development, expression patterns of Oct-3/4 are consistent with expression patterns of *Fgf-4* (Schöler, 1991; Niswander and Martin, 1992). However, in postimplantation mouse embryos, *Fgf-4* and *Oct-3/4* are expressed in distinct regions as well as in overlapping regions (Rosner et al., 1990; Niswander and Martin, 1992). This suggests that Oct-3/4 is an important regulator of Fgf-4 expression in preimplantation mammalian embryos. The *Fgf-4* null ES cells do not require Fgf-4 to proliferate in vitro, and addition of FGF-4 has little effect on their growth (Wilder et al., 1997). Furthermore, Fgf-4 null ES cells can differentiate in vitro after addition of retinoic acid. However, the survival of the differentiated ES cells is impaired. Importantly, the addition of FGF-4 to the culture medium increases the number of differentiated cells derived from the *Fgf-4* null ES cells, especially cells with many of the properties of parietal extraembryonic endoderm. Certain lineages formed in vitro are affected by the inactivation of the *Fgf-4* gene, in particular specific cells that form during the initial stage of ES cell differentiation. Kunath et al. reported that the function of FGF-4 in mouse undifferentiated ES cell was the activation of the Erk1/2 signaling cascade (Kunath et al., 2007). Inhibition of FGF or Erk activity does not disturb the expansion of undifferentiated ES cells. Instead, such treatments restrict the ability of ES cells to commit to differentiation. Disruption of Fgf-4 signaling impairs neural and mesodermal induction in ES cell. Moreover, Erk2-null ES cells fail to differentiate into either neural or mesodermal lineage, and maintain expression of pluripotency markers Oct-3/4, Nanog and Rex1. These findings indicate that FGF-4-Erk1/2 is important for neural and mesodermal commitment in ES cells. Recently, Ying et al. reported that blockage of FGF-4-ERK signaling by small molecule in-

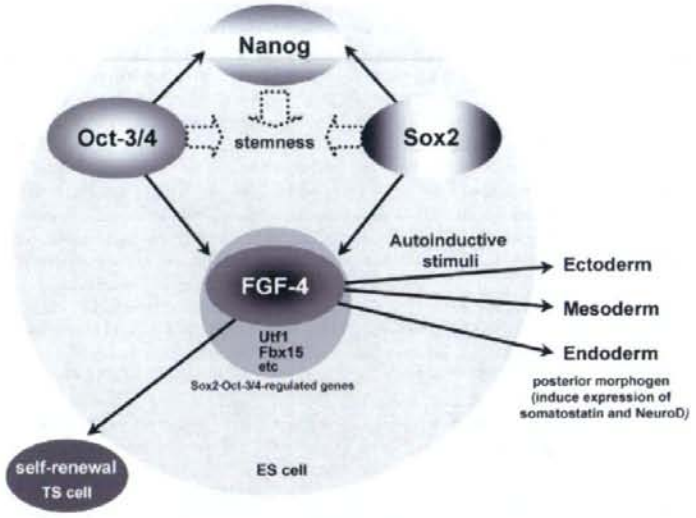

Fig. 2. Regulation and function of fibroblast growth factor-4 (FGF-4) in ES cell. The POU family transcription factor Oct-3/4 is a pivotal regulator of pluripotency in embryonic stem (ES) cells (Nichols et al., 1998). Sox2 is known to co-operate with Oct-3/4 in activating Oct-3/4 target genes (Yuan et al., 1995). ES-specific enhancers that contain binding sites for Oct-3/4 and Sox2 have been identified in several genes, including Fgf-4, Utf1 (Nishimoto et al., 1999), Fbx15 (Tokuzawa et al., 2003), and Nanog (Kuroda et al., 2005; Rodda et al., 2005). Nanog have been identified as transcription factors essential for maintaining pluripotency of ES cells in mice (Niwa, 2007). Absence of Fgf-4, progression of ES cells to either neural or mesodermal lineage commitment is arrested (Kunath et al., 2007). FGF-4 is expressed by the primitive streak and induces posterior endoderm markers (Wells and Melton, 2000). Trophoblast stem (TS) cell lines can be derived from both blastocysts and E6.5 extraembryonic ectoderm by culturing in the presence of FGF-4 (Tanaka et al., 1998). By removing FGF-4 and embryonic fibroblast conditioned medium, TS cells differentiate into various trophoblast subtypes in culture.

hibitors can maintain ES cell self-renewal (Ying et al., 2008). They used two major inhibitors for FGF receptor tyrosine kinases (SU5402) and ERK cascade (PD184352). By using a low dose of these two small-molecule inhibitors together, undifferentiated ES cell expand through multiple passages. Mayshar et al. reported that microarray analysis identified FGF-4 as a candidate for autocrine signaling in human embryonic stem (hES) cells (Mayshar et al., 2008). They indicated that FGF-4 was produced by multiple undifferentiated hES cells lines. Interestingly, undifferentiated hES cells lines also produce a FGF-4 splice isoform (FGF-4si) that codes for the amino-terminal half of FGF-4. Although FGF-4 supports the undifferentiated growth of hES cells, FGF-4si counteracts its effect by disrupting FGF-4-induced Erk1/2 phosphorylation. Expression of *FGF-4* and *FGF-4si* is detected in hES cells and early differentiated cells. Although the expression of *FGF-4* terminates in mature differentiated cells, mature differentiated hES cells maintain the expression of FGF-4si. Taken together, these reports suggest that FGF-4 is an autoinductive signal that stimulates differentiation of ES cells. Although the existence of *FGF-4si* in mouse ES cell is still unknown, it is important to show *FGF-4si* function there. Hence, it appears that if the expression profiles of *FGF-4si* are different between human and mouse ES cells, they may reflect the different characters of these cells.

Before implantation in the uterus, mammalian embryos produce trophoblast stem cells that are maintained in the extraembryonic ectoderm to develop the fetal region of the placenta. Oct-3/4 regulates autocrine growth factor signaling in ES cell precursors of trophectoderm (Nichols et al., 1998). Oct-3/4 autonomously induces expression of Fgf-4, which helps block differentiation of trophectoderm from stem cells. As shown before, the activity of Oct-3/4 is essential for the identity of the pluripotential cell population in the mammalian embryo. A culture of mouse blastocysts or early postimplantation trophoblasts in the presence of FGF-4 enables the isolation of trophoblast stem (TS) cell lines (Fig. 2; Tanaka et al., 1998). Furthermore, FGF-4 and TGF-β maintain long-term continuous TS cell proliferation (Erlebacher et al., 2004). Constitutive FGF-4 signaling in TS cells inhibits the ability of TGF-β to block c-myc expression. Furthermore, TGF-β-related protein Nodal induces FGF-4 expression in epiblast. Then Nodal and FGF-4 act directly on extraembryonic ectoderm to inhibit differentiation of trophoblast stem cells (Guzman-Ayala et al., 2004). Yang et al. reported that Shp2 was required for FGF-4-induced activation of the Src/Ras/Erk pathway (Yang et al., 2006). Depletion of the pro-apoptotic protein Bim blocks apoptosis and significantly restores Shp2 null TS cell proliferation. These results indicate that TS cells survive by FGF-4 through the Shp2/Src/Ras/Erk pathway.

Taken together, FGF-4 acts to maintain the pluripotency of ES cells and promote self-renewal of TS cells.

ROLE OF FGF-4 IN ADULT TISSUE STEM CELL

Yamamoto et al. performed a highly sensitive RT-PCR analysis to elucidate the expression of the *Fgf-4* gene in adult mice tissues (Yamamoto et al., 2000). *Fgf-4* gene expression is predominantly detected in the nervous system, intestines, and testis of normal adult mice, and is weakly recognized in other tissues such as the spleen, bone marrow, kidney, lung, eyeball, and tongue. In situ hybridization revealed cell type-specific *Fgf-4* gene expression: Purkinje cells in the cerebellum and Sertoli cells in the testis.

In our current experiments, we show that FGF-4 stimulates neural progenitor cell proliferation and induces neuronal differentiation in neurospheres, which are heterogeneous and composed of a mixed population of progenitors and stem cells (Kosaka et al., 2006). In situ hybridization re-

Developmental Dynamics

veals that the expression of *Fgf-4* mRNA is highly restricted in the subventricular zone, rostral migratory stream, and subgranular region of dentate gyrus, regions where adult neurogenesis is continuously occurring (van Praag et al., 2002). It was previously reported that FGF-4 acted as a mitogen for neural progenitor cells isolated from the fetal and adult rat central nervous system (Ray et al., 1997). Furthermore, the addition of FGF-4 increases the number of neural precursor cells that are generated from ES cells (Ying et al., 2003). Ye et al. have shown that FGF-4, which was expressed in the primitive streak, induced 5-hydroxytryptamine neurons in the ventral midbrain (Ye et al., 1998). In this regard, Shimozaki et al. reported that up-regulation of Oct-3/4 in ES cells led to neuroectoderm formation and neuronal differentiation (Shimozaki et al., 2003). Furthermore, Sox2 is expressed in the neural tube from the early stage of its formation (Zappone et al., 2000). The expression levels of Oct-3/4 are critical for their varied functions. A less than twofold increase from the normal expression level causes ES cell differentiation into ectoderm and mesoderm, whereas a reduction to less than 50% leads to their dedifferentiation into trophectoderm (Niwa et al., 2000). Oct-3/4 and Sox2 are known to cooperate in activating the transcription of *Fgf-4*, indicating a role for FGF-4 in neuronal differentiation.

Administration of adenoviruses carrying the *Fgf-4* gene or recombinant FGF-4 protein results in an increase in the platelet count. The number of megakaryocytes in the bone marrow and spleen of the animals with FGF-4 is increased compared with the control animals (Sakamoto et al., 1994). Furthermore, FGF-4 increases the count of large megakaryocytes in bone marrow, which specifically recover platelet counts in thrombocytopenic mice (Konishi et al., 1995). An in vitro study demonstrated that FGF-4 promoted megakaryocyte maturation, inducing increases in DNA ploidy, cytoplasmic and membrane maturation, and platelet-like particle release in human megakaryocytic Dami cells (Konishi et al., 1996). Moreover, FGF-4 acts on megakaryocytic cells to induce secretion of IL-6 and TNF-α, and increases adhesion of megakaryocytic cells to human endothelial cells through very late antigen-4 (VLA-4) and lymphocyte function-associated antigen 1 (LFA-1) molecules. FGF-4 stimulates the proliferation of megakaryocyte progenitors not alone but synergistically with IL-3 and with thrombopoietin. These results are also demonstrated in an in vivo study (Avecilla et al., 2004). Avecilla et al. reported that FGF-4 and SDF-1 enhanced vascular cell adhesion molecule-1 (VCAM-1)- and VLA-4-mediated localization of CXCR4-positive megakaryocyte progenitors to the vascular niche, promoting survival, maturation and platelet release in thrombocytopenic, TPO-deficient or Mpl-deficient mice. Of interest, the addition of FGF-4 to human long-term bone marrow cultures increased both the cell density of the stromal layer and the number of hematopoietic progenitor cells (Quito et al., 1996). FGF-4 supportively contributes to the development of stromal cells both from leukemic and nonleukemic marrow cells (Koh et al., 2002). These observations indicate that FGF-4 stimulates hematopoietic progenitor cell expansion through stromal cell development, although a direct effect on hematopoietic stem or progenitor cells cannot be ruled out.

The *Fgf-4* gene expresses in the testis of normal adult mice (Yamamoto et al., 2000), which suggests its possible role in spermatogenesis. Conditional transgene expression of Fgf-4 demonstrated that the specific gain of function of the *Fgf-4* gene in the testis resulted in markedly enhanced spermatogenesis (Yamamoto et al., 2002). Transgenic mice overexpressing Fgf-4 in the testis were exposed to Adriamycin, an anticancer drug causing testicular toxicity. Enhanced expression of Fgf-4 in the testis recovered the adriamycin-induced testicular damage. Furthermore, FGF-4 can act as a physiological anti-apoptotic factor for male germ cells in stimulating lactate production of Sertoli cells (Hirai et al., 2004). Apoptosis plays an important role in controlling the number of male germ cells during testicular development and spermatogenesis. Testes of adult male mice that received an adenovirus carrying human FGF-4 or a control adenovirus were exposed to mild hyperthermia, which causes germ cell apoptosis. FGF-4 significantly reduces the apoptotic death of germ cells and prevents testicular weight loss and sperm count reduction. *Fgf-4* present in mice testes is up-regulated in vivo when the testes are exposed to mild hyperthermia, and endogenous *Fgf-4* mRNA expression in Sertoli cells is also induced when the cells are exposed to mild hyperthermia in vitro. On the other hand, upon FGF-4 stimulation, lactate production from Sertoli cells was induced, which is an indispensable nutrient for germ cell survival.

Recently many reports demonstrated that FGF-4 could induce hepatocyte differentiation from ES cell or mesenchymal stem cell (Banas et al., 2007b). For instance, ES cells treated with a combination of FGF-1, FGF-4, and HGF induced an increase in hepatocytic cell numbers (Teratani et al., 2005). Multipotent adult progenitor cells (MAPCs) from bone marrow can differentiate into most mesodermal cells and neuroectodermal cells in vitro and into all embryonic lineages in vivo (Reyes et al., 2002). MAPCs not only differentiate into mesenchymal cell types, but also into endothelium, as well as cells with neuroectodermal phenotype and function. Schwartz et al. showed that MAPCs from mice, rats, or humans treated in vitro with FGF-4 and HGF not only express hepatocyte markers but also have functional characteristics consistent with hepatocyte metabolic activities (Schwartz et al., 2002). On the other hand, adipose tissue-derived mesenchymal stem cells after incubation with HGF, FGF-1, and FGF-4, specifically the CD105 positive fraction of adipose tissue-derived mesenchymal stem cells, exhibited high hepatic differentiation ability in an adherent monoculture condition (Banas et al., 2007a). Furthermore, Fgf-4, whose expression levels are undetectable in normal liver, is up-regulated in the regenerating liver after CCl_4-treatment (Teratani et al., 2005). In mouse embryogenesis, hepatocytes differentiate from the endoderm during embryonic development. Fgf-4 expressed in primitive streak-mesoderm can induce the expression of NeuroD and somatostatin in endoderm and differentiation of endoderm (Wells and Melton, 2000). Furthermore, FGF-4 induces expression of these genes in a concentration-dependent manner, implicating it as a posterior

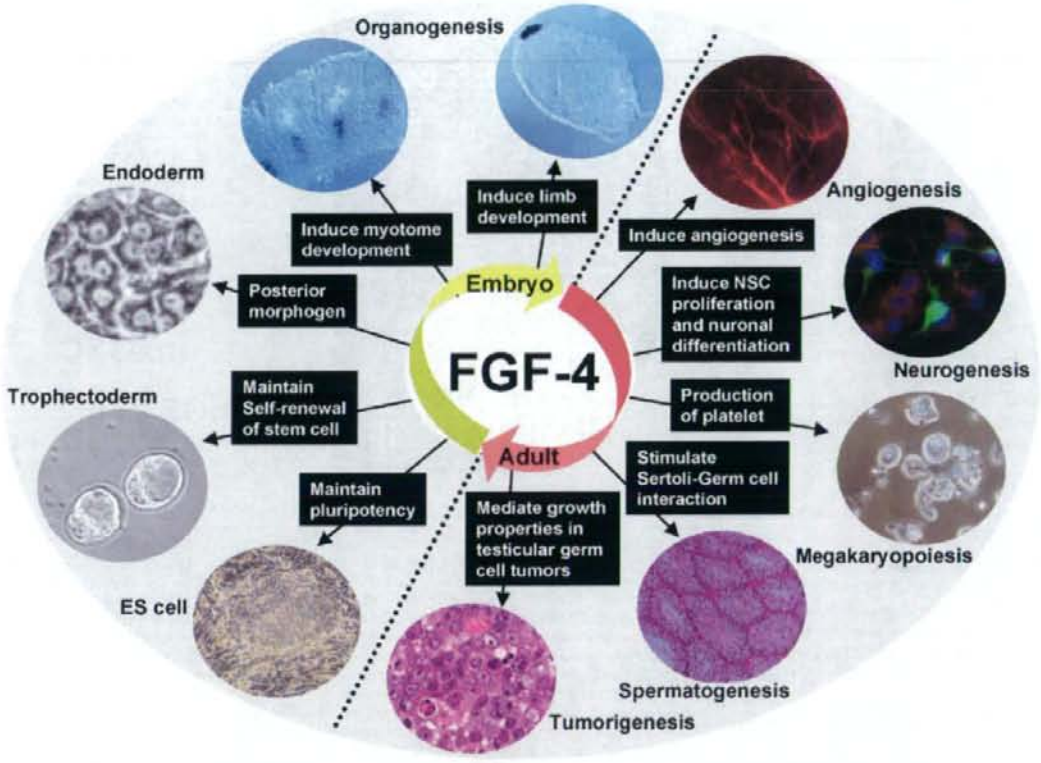

Fig. 3. Diverse functions of fibroblast growth factor-4 (FGF-4) during embryo and adult stages. FGF-4 has pleiotropic roles in many cell types and tissues; it is a mitogenic, angiogenic and survival factor, which is involved in cell proliferation and differentiation and in a variety of development processes.

morphogen. These reports indicate that FGF-4 stimulation promotes hepatocyte differentiation in ES cells, tissue-derived mesenchymal stem cells and embryogenesis. Hence, it might be interesting to study the role of FGF-4 in the differentiation of hepatic stem cells for a further understanding of the molecular mechanism of hepatogenesis.

In mouse small intestine, induction of endogenous *Fgf-4* expression was detected when mice were exposed to irradiation (Sasaki et al., 2004). Expression of *Fgf-4* is found in the epithelial cell of the villi and crypt cells. Pretreatment of FGF-4 causes an increase in the number of surviving crypt cells, and suppresses the radiation-induced apoptosis of the crypt cells. Moreover, exogenous FGF-4 enhanced epithelial cell migration and proliferation in an in vitro model. FGF-4 also has an angiogenic activity in vivo as well as in vitro (Yoshida et al., 1994). The NIH3T3 transformant transfected with the FGF-4 appeared to develop a highly vascularized tumor on nude mice. Taken together, these reports indicate that FGF-4 is a pleiotropic factor inducing many cellular functions, including angiogenesis, neurogenesis, spermatogenesis, and megakaryopoiesis (Fig. 3). However, regulation of FGF-4 and the FGF-4–evoked intracellular signaling in these cellular functions is still unclear. Verifying these mechanisms should lead to a clarification of FGF-4 controlled homeostasis in organisms.

PERSPECTIVE

Studies in the past 20 years clarified the essential role of FGF-4 as a cytokine to regulate embryogenesis. In addition, several tumors are shown to be caused by amplification of FGF-4. In this review, based on experimental observations, we have presented our views on how the FGF-4 was able to regulate stem cell fate including embryonic and somatic stem cells (Fig. 3). Finally, in this article, we wanted to emphasize the importance of FGF-4 in regulating stem cells and cancer stem cells (Fig. 4).

Most tissues and organs contain minor populations of stem cells and progenitor cells. These cells are necessary in the developing fetus for the generation of tissues and organs and later in the adult for ongoing tissue maintenance and regeneration after injury. Cancer stem cells are cancer cells that originate from the transformation of normal stem cells (Reya et al., 2001).

Developmental Dynamics

The most important property of a stem cell is its ability to self-renew. Both normal stem cells and cancer stem cells share various markers of "stemness."

The signaling pathways of Notch, Wnt, and Shh have recently been implicated in stem cell self-renewal. Notch activation promotes hematopoietic stem cell (HSC) self-renewal (Varnum-Finney et al., 2000; Karanu et al., 2000). The *Serrate-1* gene, which encodes transmembrane ligands to Notch receptors, is induced in dental mesenchyme by Fgf-4 during tooth development (Mitsiadis et al., 1997). RA induces *Fgf-4* expression in the ridge and FGF-4 subsequently activates *Shh* expression (Niswander et al., 1994). Moreover, Fgf-4 and Shh can mediate signaling from the ridge and posterior mesenchyme, respectively (Yang and Niswander, 1995). The Wnt signalling pathway has also been shown to regulate both self-renewal and oncogenesis in different organs. Wnts are involved in morphogenesis and patterning, and their proliferation-promoting roles are critical for stem cell maintenance and the expansion of progenitor pools (Willert et al., 2003; Hirabayashi et al., 2004). Overexpression of activated β-catenin (a downstream activator of the Wnt signalling pathway) in long-term cultures of HSCs expands the pool of HSCs. Cultured human keratinocytes with a higher proliferative potential have increased levels of β-catenin compared with keratinocytes with a lower proliferative capacity. However, the molecular mechanisms by which Wnt signalling and FGF-4 influence stem cells remain to be elucidated. For instance, the concerted action of FGFs and Wnts is believed to be important for inducing neural fate decisions (McGrew et al., 1997). Of interest, it was reported that the *Fgf-4* gene is a direct transcriptional target for LEF1, a nuclear mediator of Wnt signaling by association with its co-activator β-catenin. Loss of Lef1 results in inhibited tooth development at the late bud stage and LEF1 is required for a Wnt signaling to a cascade of FGF signaling activities to mediate the epithelial-mesenchymal interaction during tooth morphogenesis (Sasaki et al., 2005). However, exogenous FGF-4 can rescue the developmental arrest of

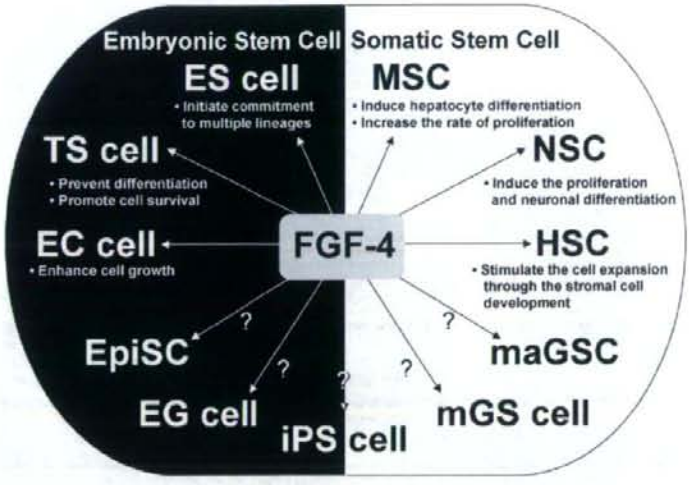

Fig. 4. Schematic description of the relationship between fibroblast growth factor-4 (FGF-4) and stem cells. Expression of *FGF-4* is restricted to undifferentiated embryonic stem (ES) cells and embryonal carcinoma (EC) cell lines (Yoshida et al., 1988; Velcich et al., 1989; Schoorlemmer and Kruijer, 1991). FGF-4 enhances EC cell growth (Maerz et al., 1998) and ES cells lacking FGF-4 resist neural and mesodermal induction (Kunath et al., 2007). FGF-4 permitted the isolation of permanent trophoblast stem (TS) cell lines (Tanaka et al., 1998). Exogenous FGF-4 increases the rate of mesenchymal stem cell (MSC) proliferation (Farré et al., 2007). Furthermore, FGF-4 could induce hepatocyte differentiation from MSC (Banas et al., 2007). FGF-4 stimulates hematopoietic stem/progenitor cells expansion through the stromal cell development (Quito et al., 1996; Koh et al., 2002). Moreover, FGF-4 stimulates neural stem/progenitor cell proliferation and induces neuronal differentiation (Kosaka et al., 2006). The expression of *FGF-4* in induced pluripotent stem (iPS) cell derived from mouse and human was confirmed (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). There have been no reports that address the expression of *FGF-4* in other stem cells, including EpiSC (epiblast-derived stem cells), EG (embryonic germ) cells, GCS, and maGSC (multipotent adult germline stem cells). However, FGF-4 gene is not expressed in mGS (multipotent germline stem) cells (Imamura et al., 2006).

Lef1−/− tooth germs (Kratochwil et al., 2002). It seems likely that FGF-4 regulates stem cells and cancer stem cells in conjunction with other molecules known to be important in this process. It will also be important to determine whether the Wnt, Notch, Shh, and FGF-4 pathways interact to regulate stem and progenitor cell self-renewal. If these signalings are dysregulated, these pathways could contribute to oncogenesis. There are a few reports that described the identification of FGF-4 induced gene expression. Guthridge et al. isolated the 21 cDNA of late-induced genes from a FGF-4 transformed NIH3T3 cell line. This study indicated that FGF-4 induced a wide range of genes including cyclin D1, HSP-90, LAMP-1 and p63 (Fig. 1; Guthridge et al., 1996). Overexpression of cyclin D1 is known to correlate with the early onset of cancer and tumor progression (Fu et al., 2004). Moreover, p63 is an essential regulator of stem-cell maintenance in stratified epithelial tissues (McKeon, 2004). Thus, these results suggested the indirect association of FGF-4 with stem cells and cancer development.

Recently, pluripotent stem cells—which are called induced pluripotent stem (iPS) cells, produced from adult mouse and human fibroblasts by introducing four factors, Oct-3/4, Sox2, c-Myc, and Klf4—were established (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). In iPS cells, expression of FGF-4 was confirmed as the marker for a pluripotent stem cell. However there are no reports that describe the function of FGF-4 in iPS cells. It is very interesting why these artificially induced pluripotent stem cells express FGF-4, which is the autoinductive stimuli in ES cells. There are several pluripotent stem cells that have been established other than iPS

cells. For example, FGF-2, in the presence of stem cell factor (SCF) and LIF, stimulates long-term proliferation of primordial germ cells (PGCs; Matsui et al., 1992; Resnick et al., 1992). These embryonic germ (EG) cells resemble embryonic stem cells. Moreover, two groups have reported a new stem cell line, derived from the epiblast, a tissue of the postimplantation embryo that generates the embryo (Tesar et al., 2007; Brons et al., 2007). These cells, which are referred to as EpiSCs (postimplantation epiblast-derived stem cells), express Oct-3/4, Nanog and Sox-2. Kanatsu-Shinohara et al. established ES-like cells, called mGS (multipotent germline stem), from neonatal mouse testis (Kanatsu-Shinohara et al., 2004). These ES-like cells are phenotypically similar to ES and EG cells. Furthermore, Guan et al. showed the isolation of spermatogonial stem cells (SSCs) from adult mouse testis (Guan et al., 2006). These isolated SSCs acquire embryonic stem cell properties. These cells are called multipotent adult germline stem cells (maGSCs). There is no strong evidence supporting a relationship between FGF-4 and these stem cells. However, the impact of FGF-4 on ES cell and TS cell suggests that FGF-4 has a function in regulating the stemness of these newly established stem cells.

The diverse function of FGF-4 is partly regulated by (1) specificity of ligand–receptor interactions in FGFR signaling and (2) by transcriptional factor (i.e., Oct-3/4 and Sox2). FGF signaling uses receptor tyrosine kinases that form high-affinity complexes with FGFs and heparan sulfate proteoglycans at the cell surface. Whereas FGF-2 binds heparan sulfate ubiquitously, FGF-4 exhibits a restricted pattern, failing to bind heparan sulfate in the heart and blood vessels and failing to activate signaling in embryonic day-18 mouse aortic endothelial cells. This suggests that FGF-4 seeks a specific heparan sulfate sulfation pattern, distinct from that of FGF-2, which is not expressed in most vascular tissues. This in turn suggests that FGF and FGFR recognition of specific heparan sulfate sulfation patterns is critical for the activation of FGF signaling, and that synthesis of these patterns is regulated during embryonic development (Allen et al., 2001). Urakawa et al. reported that a previously undescribed receptor conversion by Klotho, a senescence-related molecule, generates the FGF-23 receptor (Urakawa et al., 2006). They showed that the Klotho and FGFR1 (IIIc) reconstitute the FGF-23 receptor. These findings suggest the existence of a novel mechanism of interactions between FGF and FGFRs. Because mitogenic activity of FGF-4 and FGF-2 is similar, unidentified mechanisms for intracellular signaling regulate the different biological activity of FGF-4 and FGF-2. West et al. reported that neuropilin-1 (Npn-1), which is a receptor for semaphorin in the nervous system and interacts with the heparin binding isoforms of VEGF in endothelium, interacts with FGF-4 (West et al., 2005). This result raises the possibility that Npn-1 modulates FGF-4 activity. FGF-4 regulates various cell functions at a local level. In this regard, because the expression of FGF-4 is very low in a somatic organ, the gene could be regulated by transcription factors other than Oct-3/4 and Sox2.

Compelling insights into the molecular framework by which FGF-4 promotes several biological processes have been discovered, and further functional analysis based on well-defined models and more elaborate system models defining genetic responses of stem cells will contribute to the understanding of the novel function of FGF-4 in controlling the fate of embryonic and somatic stem cells.

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Activation-Induced Cytidine Deaminase Links Between Inflammation and the Development of Colitis-Associated Colorectal Cancers

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See editorial on page 736.

Background & Aims: **Activation-induced cytidine deaminase (AID) was originally identified as an inducer of somatic hypermutations in the immunoglobulin gene. We recently revealed that ectopic AID expression serves as a link between the cellular editing machinery and high mutation frequencies, leading to human cancer development. In the current study, we investigated whether AID might contribute to the development of colitis-associated colorectal cancers.** ***Methods:*** **The expression and regulation of AID in association with proinflammatory cytokine stimulation were investigated in cultured colonic cells. Genotoxic activity of AID in colonic cells was analyzed using retroviral system. Immunohistochemistry for AID was carried out on various human colonic tissues specimens.** ***Results:*** **Tumor necrosis factor-α induced aberrant AID expression via IκB kinase-dependent nuclear factor (NF)-κB-signaling pathways in human colonic epithelial cells. Moreover, AID expression was also induced in response to the T helper cell 2-driven cytokines interleukin-4 and interleukin-13, which are activated in human inflammatory bowel disease. Aberrant activation of AID in colonic cells preferentially induced genetic mutations in the *TP53* gene, whereas there were no nucleotide alterations of the *APC* gene. Immunohistochemistry revealed enhanced expression of endogenous AID protein not only in the inflamed colonic mucosa of ulcerative colitis patients but also in tumor lesions of colitis-associated colorectal cancers.** ***Conclusions:*** **Our findings indicate that proinflammatory cytokine-mediated aberrant expression of AID in colonic epithelial cells is a genotoxic factor linking inflammation, somatic mutations, and colorectal cancer development.**

Chronic inflammatory bowel diseases (IBD) are important etiologic factors in the development of colorectal cancers.[1] A cohort study of patients with ulcerative colitis (UC) revealed that extensive colitis increases the cumulative risk of colorectal cancer by 7.2% at 20 years and 16.5% at 30 years from disease onset.[2] Thus, the relative risk of colorectal cancer in patients with UC was up to 20 times higher than that of the general population.[3] Colon cancers arising in IBD patients have several distinct characteristics compared with sporadic colorectal cancers. There is a higher rate of multiple synchronous cancers and dysplastic lesions associated with cancer development.[4] Mutations in the *TP53* gene appear to be an early event and are already present in dysplastic lesions associated with UC.[5,6] These findings suggest that chronic inflammation of the colonic mucosa has a critical role in colon carcinogenesis, and the molecular processes leading to colitis-associated cancer development might differ from those of sporadic colorectal cancers. However, the mechanisms that account for the development of colon cancers via chronic inflammation remain unclear.

Recently, we demonstrated the possible role of activation-induced cytidine deaminase (AID) in linking chronic inflammation to the development of human gastric and liver cancers.[7–9] Under physiologic conditions, AID is required for somatic hypermutation and class switch recombination in immunoglobulin genes of activated B cells.[10,11] However, the inappropriate expression of AID could contribute to tumorigenesis via its DNA mutagenic activity.[12] In fact, constitutive and ubiquitous expression of AID in mice causes the development of neoplastic lesions including cancers in several organs in association with high mutation frequencies.[8,9,13,14] Notably, ectopic AID expression is induced in response to tumor necrosis factor α (TNF-α), a proinflammatory cytokine that is important in the pathway leading to tumorigenesis.[15] These findings demonstrate a novel linkage between in-

Abbreviations used in this paper: AID, activation-induced cytidine deaminase; IKK, IκB kinase; IL, interleukin; NF-κB, nuclear factor κB; PCR, polymerase chain reaction; siRNA, small interfering RNA; STAT, signal transducers and activators of transcription; TCR, T-cell receptor; TNF, tumor necrosis factor.

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flammation and enhanced susceptibility to somatic mutations leading to tumor development in gastric epithelial cells and hepatocytes.

Excessive and chronically produced proinflammatory mediators are thought to contribute to tumor promotion and progression in colitis-associated cancers.[16] Expression of most proinflammatory cytokines and chemokines, including TNF-α, is up-regulated in the colonic tissues of patients with IBD,[17] suggesting that enhanced proinflammatory cytokine activity contributes to colitis-associated cancer development. Based on these findings, we speculated that AID might be involved in colon carcinogenesis within the background of chronic colitis, and we therefore investigated the proinflammatory cytokine stimulation-induced expression and regulation of endogenous AID in human colonic epithelial cells.

Materials and Methods

Plasmids and Reagents

The expression plasmids pcDNA3-IκB kinase (IKK) α, IKKβ, and RelA (nuclear factor [NF]-κB) were as described.[18] The expression plasmids pcDNA3-IκB$\alpha\Delta$N, encoding the super-repressor form of IκBα, and pcDNA3-IKKβ (K44A), encoding the dominant negative mutant of IKK-β have also been described.[8] Expression plasmid encoding the dominant negative form of STAT6 (STAT6ΔC) lacking the C-terminal 186 amino acids[19] was generated from the STAT6 fragment using polymerase chain reaction (PCR) amplification. Recombinant human TNF-α, interleukin (IL)-4, IL-13, IL1-β, IL-12, and interferon (IFN)-γ were obtained from PeproTech EC Ltd. (London, United Kingdom).

Quantitative Real-Time Reverse-Transcription PCR

Quantitative real-time reverse-transcription (RT) PCR (RT-PCR) for human *AID* amplification was performed using a 7300 Real-Time PCR System (PE Applied Biosystems, Foster City, CA). The 6-carboxyfluorescein (FAM)-labeled probe used for human *AID* was 5′-TCGGCGTGAGACCTACCTGTGCTAC-3′. Target complimentary DNA (cDNA) were normalized to endogenous messenger RNA (mRNA) levels of the housekeeping reference gene *18s ribosomal RNA (18s rRNA)*.[7] For simplicity, ratios are represented as relative values compared with expression levels in a lysate from control cells. The reproducibility of this quantification method was examined by comparing results obtained from replicate samples during the same reaction run with those from independent runs on different days. The PCR procedures were performed at least 3 times for each sample.

Cell Culture and Transfection

LoVo cells were cultured in Ham's F12 (MP Biomedicals, Solon, OH) containing 10% fetal bovine serum. SW48 human colonic cancer cells were grown in Dulbecco's modified Eagle medium (Gibco-BRL, Rockville, MD). Transfection of plasmids was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Small interfering RNA (siRNA) duplexes used for targeting signal transducers and activators of transcription (STAT) 6 were obtained from Invitrogen.

Recombinant Retrovirus Production and Infection of Colon Cancer Cells

The retroviral system for expression of AID was performed as described.[20] A full-length AID cDNA was subcloned into the *Eco*RI and *Xho*I restriction sites of the pFB vector (Stratagene, La Jolla, CA) with internal ribosome entry and a puromycin resistance gene. Viral vectors for the expression of the mutant AID (R35E, R35E/R36D) were constructed as described.[21]

NF-κB Luciferase Reporter Gene Assay

Luciferase assays were performed using the Dual-Luciferase TM Reporter assay system (Promega, Madison, WI). The transfection efficiency was normalized to cells cotransfected with pRL vector (Promega).[18]

Subcloning and Sequencing of Tumor-Related Genes

The oligonucleotide primers for the amplification of the human *TP53, APC, K-ras, and c-myc* genes are shown in Supplementary Table 1 (see Supplementary Table 1 online at www.gastrojournal.org). Amplification of the target sequences was performed using high-fidelity Phusion Taq polymerase (Finnzymes, Espoo, Finland), and the products were subcloned into a pcDNA3 vector (Invitrogen). The resulting plasmids were subjected to sequence analysis.

Immunoblotting and Immunohistochemistry

A polyclonal antibody against human AID was generated using purified recombinant AID protein as an immunogen.[22] A mouse monoclonal antibody against α-tubulin was purchased from Calbiochem (San Diego, CA), and a rabbit monoclonal antibody against human phospho-STAT6 was purchased from Cell Signaling Technology (Danvers, MA). Immunohistochemistry was performed as described protocol.[23]

Patients

The study group consisted of patients who had undergone colectomy because of severe UC or colitis-associated colon cancers at Dokkyo University or Kyoto University Hospitals between 2003 and 2005. Selection of patients enrolled in this study was based on the availability of a sufficient amount of tissue for analysis. The patients included 6 men and 6 women, with an average age at the time of surgery of 45.3 $\pm$ 12.4 years (mean $\pm$ SD; range, 22–72 years). As a control, 5 samples of nor-

mal colon tissues from the nontumorous region of patients with sporadic colon cancers were also examined. Biopsies of tumor tissue at the proximal edge of freshly resected specimens were obtained and immediately frozen in liquid nitrogen. Written informed consent for the use of resected tissue was obtained from all patients in accordance with the Declaration of Helsinki, and the Kyoto University Graduate School and Faculty of Medicine Ethics Committee approved the study.

Results

Human AID Is Induced in Response to TNF-α Signaling in Colonic Epithelial Cells

To gain preliminary insight into the role of human AID proteins in human colonic epithelial cells, the expression of AID was analyzed by quantitative RT-PCR in cultured human colon cancer cells. We first confirmed that *cIAP1*, a TNF-α-inducible gene in many types of cells, was increased in response to TNF-α stimulation in colonic cells (Figure 1*A*). Endogenous AID expression was markedly elevated after TNF-α treatment in both LoVo and SW48 cells (Figure 1*A* and *B*). TNF-α induced a time-dependent transcriptional up-regulation of *AID* with a peak level 8 to 12 hours after treatment, whereas expression of the internal control *18S rRNA* transcript was unchanged (Figure 1*C*). Immunoblotting analysis using a specific antibody against human AID revealed that TNF-α induced a time-dependent up-regulation of AID protein in both cell types, with a peak level 12 to 24 hours after treatment (Figure 1*D* and *E*). RT-PCR analysis revealed that *AID* transcripts also increased in response to another proinflammatory cytokine, IL-1β, in LoVo cells (see Supplementary Figure 1*A* online at www.gastrojournal.org). Taken together, these findings suggest that endogenous AID expression is induced by proinflammatory cytokine stimulation in human colonic cells.

NF-κB Mediates AID Expression in an IκB Kinase-Dependent Manner in Colonic Epithelial Cells

Transcription factor NF-κB is induced by TNF-α signaling, and NF-κB is frequently and constitutively activated in the colonic epithelia of patients with IBD.[24] Therefore, we examined whether AID expression would be regulated transcriptionally by NF-κB activity in cultured colonic cells. First, we examined whether induction of positive regulators of NF-κB signaling affected AID expression. A reporter plasmid assay revealed enhanced NF-κB activity in cells producing the wild-type IκB kinase (IKK)-α, IKK-β, or NF-κB (Figure 2*A*). Under these experimental conditions, the expression of these NF-κB-positive regulators substantially increased the expression of endogenous AID protein in LoVo cells (Figure 2*B*). Next, we examined whether negative regulators of NF-κB, the mutant IKK-β and the super-repressor form of IκB-α

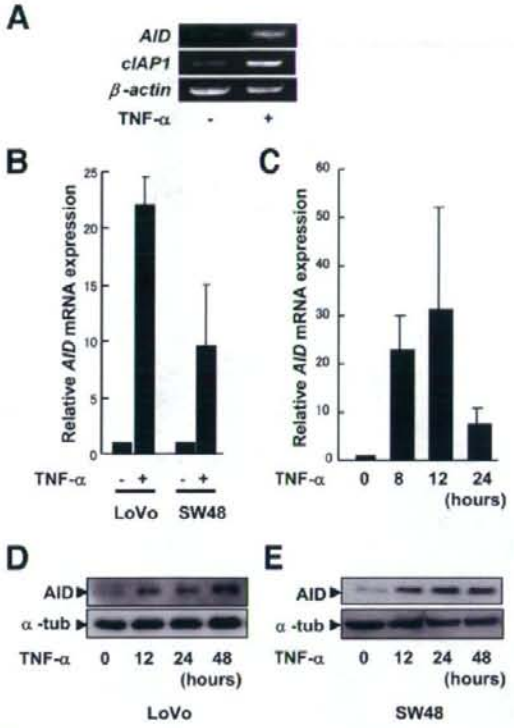

Figure 1. AID expression is induced in response to TNF-α stimulation in human colon cancer cells. (*A*) Total RNA was extracted from LoVo cells 12 hours after TNF-α treatment (100 ng/mL). Semiquantitative RT-PCR was performed using each RNA sample as a template and oligonucleotide primers specific for human *AID* (*upper panel*), *cIAP1* (*middle panel*), and *β-actin* (*lower panel*). (*B*) Total RNA was isolated from LoVo and SW48 cells before and 12 hours after stimulation with TNF-α (100 ng/mL). Real-time RT-PCR was performed using FAM-labeled probes and primers specific for human *AID*. *Values* shown in the *graphs* are normalized relative to specimens without TNF-α treatment (mean ± SD; n = 3). (*C*) Total RNA from LoVo cells was isolated immediately before and 8, 12, and 24 hours after TNF-α treatment (100 ng/mL). (*D*) LoVo and (*E*) SW48 cells were treated with TNF-α (100 ng/mL) for 0, 12, 24, and 48 hours. Total protein was isolated, and immunoblot analysis was performed using anti-human AID (*upper panels*) or anti-α-tubulin (*α-tub, lower panels*).

(IκBαΔN), would reduce AID production. We confirmed by reporter plasmid assay that TNF-α-mediated NF-κB activity was almost completely abolished by coproduction of IκBαΔN (Figure 2*C*, *upper graph*). Up-regulation of AID after TNF-α treatment was reduced substantially in LoVo cells in which IκBαΔN was coproduced (Figure 2*C*, *lower graph*). The coproduction of mutant IKK-β or IκBαΔN also reduced endogenous AID protein expression after TNF-α stimulation in both cells (Figure 2*D* and *E*). Because protein kinase Cξ is required for NF-κB activation in several cells,[25] we also examined its involvement in TNF-α-mediated AID expression. We found that

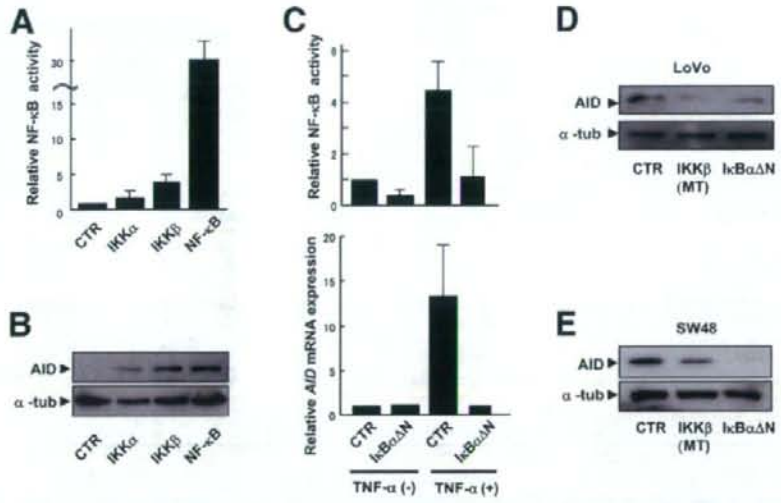

Figure 2. TNF-α-induced AID expression is achieved through the activation of NF-κB. (*A*) LoVo cells were transfected with NF-κB reporter plasmids together with expression plasmids encoding wild-type IKK-α, IKK-β, and RelA (NF-κB) or a control vector (CTR). After 48 hours, whole cell lysates were prepared, and luciferase activity was monitored in each extract. (*B*) Cell lysates were subjected to immunoblotting using anti-AID (*upper panel*) or anti-α-tubulin (*α-tub, lower panel*). (*C*) LoVo cells were transfected with a plasmid for the expression of the super-repressor form of IκB-α (IκBαΔN) or with a control vector (CTR), followed by treatment with TNF-α (100 ng/mL) for 12 hours. Each sample was harvested, and luciferase activity was measured to quantify endogenous NF-κB activity (*upper graphs*). Total RNA from each sample was isolated, and quantitative RT-PCR of endogenous *AID* expression was performed using each specimen as a template (*lower graphs*). The data present the means of *AID* mRNA expression relative to the internal control *18S rRNA* (mean ± SD; n = 3). (*D*) LoVo cells and SW48 (*E*) cells were transfected with plasmids encoding dominant negative IKKβ (IKKβMT), IκBαΔN, or a CTR vector and then treated with TNF-α (100 ng/mL) for 24 hours. Cell lysates were immunoblotted with anti-AID antibody (*upper panel*) and anti-α-tubulin (*α-tub, lower panel*).

knocking down the expression of endogenous protein kinase Cξ did not cause any significant changes in the levels of TNF-α-mediated AID expression in LoVo cells (see Supplementary Figure 1C online at www.gastrojournal.org).

These findings indicate that AID expression in colonic epithelial cells is regulated through the IKK-dependent NF-κB-signaling pathway, suggesting a common mechanism for the regulation of AID gene expression in human epithelial cells in the colon, stomach, and liver under inflammatory conditions.[8,9]

T Helper Cell 2 Cytokines IL-4 and IL-13 Are Involved in the Regulation of AID Expression in Colonic Epithelial Cells

In B cells, AID is regulated by IL-4 signaling in a STAT6-dependent manner,[26] suggesting that AID expression can be induced in human colonic epithelia by various inflammatory mediators produced in the human colon. Because IL-4 is involved in the T helper cell (Th) 2 cytokine response, which has a pivotal role in the pathogenesis of UC,[27] we examined whether IL-4 would contribute to the regulation of AID expression in colonic epithelial cells. Quantitative RT-PCR revealed marked up-regulation of *AID* transcripts in response to IL-4 treatment in both LoVo and SW48 cells (Figure 3*A*). *AID* transcripts were induced promptly, peaking at 12 to 14 hours after IL-4 treatment (Figure 3*B*). IL-4 activates STAT6 in a phosphorylation-dependent manner and contributes to the regulation of expression of various genes.[28] Immunoblotting analysis revealed that IL-4 treatment resulted in an increase in phosphorylated STAT6 protein expression (Figure 3*C* and 3*D*). Under these conditions, the expression of AID protein was increased substantially, peaking at 24 to 36 hours after IL-4 stimulation in both LoVo cells (Figure 3*C*) and SW48 cells (Figure 3*D*). We next examined whether AID expression would be mediated by a STAT6-dependent mechanism using the dominant negative form of STAT6 (STAT6ΔC) (Figure 3*E*). We found that STAT6ΔC suppressed IL-4-mediated AID expression and that coexpression of the wild-type STAT6 reversed the suppression of AID expression caused by STAT6ΔC in colonic cells (Figure 3*F*).

Another Th2 cytokine, IL-13, is a critical mediator of mucosal inflammation and could be a key molecule in the pathogenesis of human UC.[29] IL-13 shares many functional properties with IL-4, such as a common receptor subunit, the α-subunit of the IL-4 receptor, and

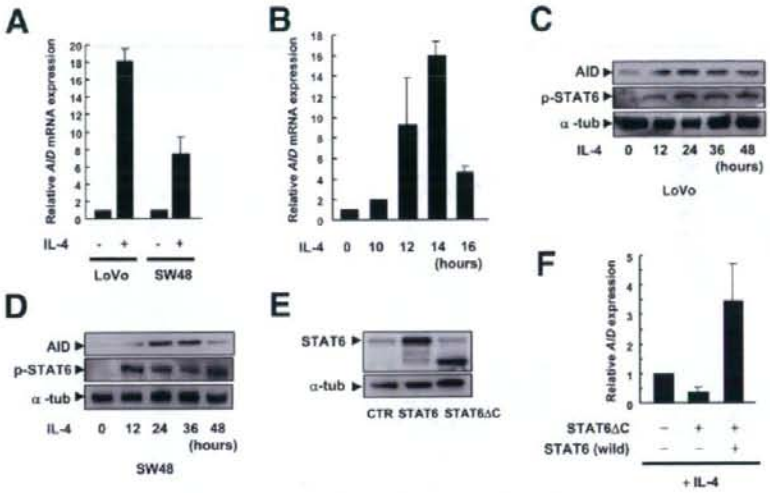

Figure 3. IL-4-mediated AID expression in human colonic epithelial cells. (*A*) LoVo and SW48 cells were treated with human recombinant IL-4 (100 ng/mL) for 12 hours. Quantitative RT-PCR was performed using FAM-labeled probes specific for human *AID*. (*B*) Time course of changes in *AID* mRNA expression after IL-4 stimulation. SW48 cells were harvested and subjected to total RNA isolation immediately before (0) and 10, 12, 14, and 16 hours after stimulation by IL-4 (100 ng/mL). (*C* and *D*) LoVo (*C*) and SW48 (*D*) cells were treated with human IL-4 (100 ng/mL) for the indicated times. Total protein was isolated, and immunoblot analysis was performed using anti-human AID (*upper panel*), anti-phospho-STAT6 (*p-STAT6, middle panel*), or anti-α-tubulin antibodies (*α-tub, lower panel*). (*E*) LoVo cells were transfected with expression plasmids encoding wild-type STAT6, STAT6ΔC, or control vector (CTR). After 48 hours, cell lysates were subjected to immunoblotting using anti-STAT6 (*upper panel*) or anti-α-tubulin (*α-tub, lower panel*). (*F*) LoVo cells were transfected with STAT6ΔC expression plasmid or control vector, followed by the cotransfection with pcDNA3-STAT6 encoding wild-type STAT6. Total RNA from each sample was isolated after IL-4 stimulation for 14 hours, and quantitative RT-PCR of endogenous *AID* expression was performed using each specimen as a template.

activates STAT6 by phosphorylation for further signal transduction.[30] Our finding that AID expression is regulated by IL-4 prompted us to test whether IL-13 would be also involved in the regulation of AID expression. Quantitative RT-PCR analysis clearly showed that IL-13 stimulation in both LoVo and SW48 cells substantially up-regulated *AID* transcripts (Figure 4*A*). A time course immunoblotting analysis revealed that AID protein expression in these cells increased in response to IL-13 and peaked at 24 hours after treatment (Figure 4*B* and *C*). Transfection of STAT6-specific siRNA, but not control siRNA, substantially suppressed phosphorylated STAT6 protein levels in LoVo cells. Under these conditions, IL-13 failed to induce AID expression in the cells treated with siRNA specific for STAT6 (Figure 4*D*). These findings support our conclusion that AID expression in human colonic epithelial cells is regulated by 2 Th2 cytokines, IL-4 and IL-13, through a STAT6-dependent pathway.

We also examined whether Th1 cytokines could trigger the expression of AID in colonic epithelial cells. We found that AID up-regulation was induced by treatment with IL-12, but not by IFN-γ, suggesting that some of the Th1 cytokines may also play roles in the aberrant expression of AID in colonic cells (see Supplementary Figure 1*A* online at www.gastrojournal.org).

AID Expression in Colonic Epithelial Cells Results in the Accumulation of TP53 Mutations

To clarify whether aberrant AID expression might be genotoxic in human colonic cells, we examined whether AID expression would cause somatic mutations in tumor-related genes. For this purpose, the mutagenetic activity of AID was determined by a retroviral vector-mediated AID expression system in LoVo cells. We investigated the overall mutation frequency in the *TP53*, *APC*, and *K-ras* genes because mutations of these genes are closely associated with the development of human colorectal cancers. In addition, we investigated the mutation occurrence in the *c-myc* gene, which is a target of AID for abnormal editing in cultured human hepatoma-derived cells.[8] Accordingly, multiple clones were picked randomly from cells at 2, 4, and 8 weeks after AID expression and subjected to sequence analysis. We first confirmed that either no changes or only a single nucleotide alteration was detected in all genes of 40 randomly picked clones from cells transfected with control vectors (data not shown). In contrast, nucleotide alterations in the *TP53* gene emerged in a time-dependent manner in cells producing AID, and a substantially higher number of nucleotide alterations appeared in cells 8 weeks after

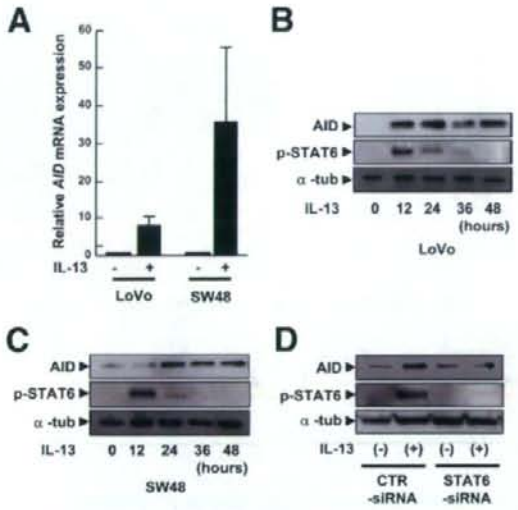

Figure 4. AID expression is regulated by IL-13 in human colonic epithelial cells. (*A*) Total RNA was isolated from LoVo and SW48 cells after treatment with human recombinant IL-13 (100 ng/mL) for 12 hours. The expression levels of *AID* mRNA were measured by quantitative real-time PCR using FAM-labeled probes specific for human *AID*. (*B* and *C*) Time course of changes in AID protein expression after IL-13 stimulation. LoVo (*B*) and SW48 (*C*) cells were treated with human IL-13 (100 ng/mL) for 0, 12, 24, 36, and 48 hours, followed by immunoblotting using anti-human AID (*upper panel*), anti-phospho-STAT6 (*p-STAT6, middle panel*), or anti-α-tubulin antibodies (*α-tub, lower panel*). (*D*) LoVo cells were transfected with siRNA targeting STAT6 or control RNA (CTR) for 24 hours, and lysates were prepared from siRNA-treated cells after stimulation with IL-13 (100 ng/mL) for 24 hours. Immunoblotting was performed using antibodies specific for human AID (*upper panel*), phospho-STAT6 (*p-STAT6, middle panel*), or α-tubulin (*α-tub, lower panel*).

AID activation, compared with control cells (Table 1). The nucleotide alterations induced by AID activation were distributed across the entire transcribed region of the *TP53* gene (Figure 5*B* and *C*). Notably, 6 of 10 nucleotide alterations that emerged in the *TP53* coding region resulted in amino acid substitutions with potential functional consequences. Compiled data in the International Agency for Research on Cancer *TP53* Mutation Database

Table 1. AID-Induced Mutagenesis in Various Tumor-Related Genes in LoVo Cells

Target gene	Duration of AID activation	Mutated clones (n/total)	Mutation number (n/total bases)
TP53 (exons 2–6)	2 weeks	1/40	1/22,000
	4 weeks	2/47	2/25,850
	8 weeks	6/48	7/26,400
TP53 (exons 6–11)	2 weeks	1/43	1/23,650
	4 weeks	2/34	2/18,700
	8 weeks	3/38	3/20,900
APC	8 weeks	0/42	0/23,425
K-ras	8 weeks	0/40	0/18,760
c-myc	8 weeks	1/41	1/21,695

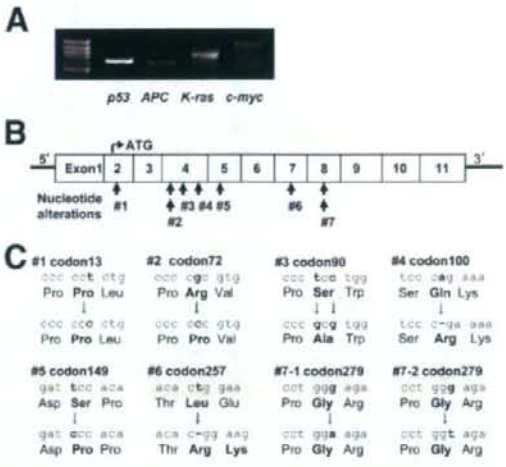

Figure 5. Distribution of mutations in the *TP53* sequence in AID-expressing LoVo cells. (*A*) Total RNA was extracted from LoVo cells and semiquantitative RT-PCR analyses were performed by using specific primers for *TP53*, *APC*, *K-ras*, and *c-myc*. (*B* and *C*) LoVo cells were infected with a retroviral vector for AID expression and cultured for 8 weeks. (*B*) Eight point mutations (numbers 1–3, 5, 7) and 2 deletions (numbers 4 and 6) appeared in the 48 (exons 2–6) and 38 (exons 6–11) sequenced *TP53* clones of LoVo cells with AID activation. (*C*) Point mutations that emerged in the coding region of the *TP53* gene resulted in amino acid substitutions.

(http://www-p53.iarc.fr/index.html) revealed that 5 of the 10 nucleotide positions altered in the *TP53* gene after AID activation corresponded to alterations observed in clinical specimens of human malignancies. In contrast to the *TP53* gene, no somatic mutations appeared in the coding sequences of the *APC and K-ras* genes, even 8 weeks after AID activation. In the *c-myc* gene, only a single nucleotide alteration was observed in the presence of AID expression. A previous study demonstrated that N-terminal mutants R35E and R35E/R36D appear less processive and have altered mutational specificity compared with wild-type AID.[21] In colonic cells, R35E and R35E/R36D-AID induced *TP53* mutations less frequently than wild-type AID expression (see Supplementary Table 2 online at www.gastrojournal.org). In addition, the *TP53* gene mutation patterns observed in the R35E-AID-expressing cells did not show any target base preferences, an observation similar to that for the *TP53* gene mutations induced by wild-type AID expression in LoVo cells. Taken together, these findings suggest that aberrant AID expression preferentially induced nucleotide alterations in the *TP53* gene in human colonic epithelial cells.

Expression of Endogenous AID Protein in UC Mucosa and Colitis-Associated Cancers

To clarify the expression and localization of AID protein in human colonic tissues under physiologic and pathologic conditions, immunohistochemistry was per-

formed using a specific antibody against human AID in colonic tissue from 22 UC lesions, 15 colitis-associated neoplasms, and 5 nontumorous regions of the patients with sporadic colon cancers. Specificity of the antibody in immunostaining was confirmed by control staining performed on germinal centers of human mesenteric lymph nodes containing mostly activated B cells.[7,9] In normal colonic mucosa lacking inflammation, immunohistochemistry revealed no evidence of AID expression (Figure 6*A*). By contrast, endogenous AID immunoreactivity was detected in the colonic epithelium of 12 of 22 (54%) inflammatory lesions from patients with UC (Figure 6*B–D*). AID immunostaining was localized mainly in the cytoplasm of inflamed colonic epithelial cells. Strongly AID-positive cells were also observed in the lymphocytes that infiltrated the submucosa of colonic tissue from patients with UC. In the colitis-associated colon cancer tissues, AID protein expression was observed in neoplastic cells in 12 of 15 tumor lesions examined (Figure 7). Interestingly, tumor lesions with AID expression also exhibited staining for TP53 protein. Because the inflamed colon of patients with Crohn's disease is at risk for developing colon cancer, we also examined whether aberrant AID expression would be present in the inflamed colonic epithelium of 8 patients with Crohn's disease. Immunostaining for AID protein was detected in the inflamed regions of the colonic epithelium in all cases examined (see Supplementary Figure 2*A* online at www.gastrojournal.org). In the case of sporadic colon cancers, AID immunoreactivity was detected in 2 of 5 cancer tissue specimens examined (see Supplementary Figure 2*B* online at www.gastrojournal.org).

To test further for AID expression in inflamed colonic mucosa, we analyzed endogenous AID expression levels in the colon of T-cell receptor (TCR) α mutant mice, an in vivo model of UC.[31] We found that AID transcript levels were up-regulated substantially in the inflamed colonic mucosa of the TCR-α mutant mice (see Supplementary Figure 3 online at www.gastrojournal.org). These findings further supported the in vitro findings that inflammatory stimulation induced aberrant AID expression in colon epithelial cells.

Discussion

Emerging evidence suggests that human carcinogenesis is a multistage process resulting from the accumulation of genetic alterations.[32] Our recent studies highlighted the importance of the DNA editor, AID, in the cellular events leading to genetic mutations during the development of inflammation-associated human cancers.[8,9] Here, we demonstrated for the first time that the Th2 cytokines IL-4 and IL-13 can induce aberrant AID expression in human colonic cells, leading to the preferential accumulation of genetic mutations in the tumor suppressor gene *TP53*. Moreover, we detected endogenous AID expression in inflamed colonic mucosa of patients with UC and in colitis-associated colorectal neoplasms. Our findings suggest that proinflammatory cytokine-mediated AID expression has a key role in generating colonic mucosa *TP53* mutations underlying IBD,

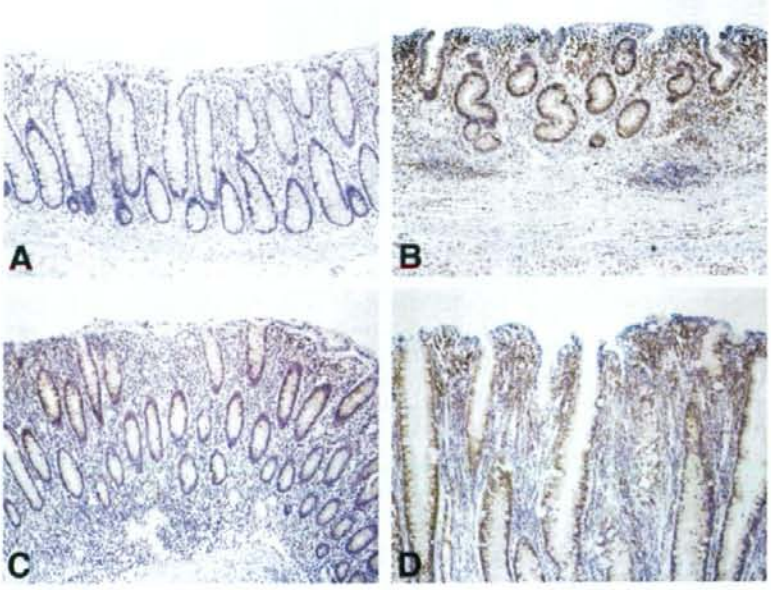

Figure 6. Expression of AID protein in UC tissue specimens. (*A*) No AID expression was observed in normal colonic tissue. (*B–D*) Strong AID immunoreactivity was present in colonic epithelial cells from patients with UC. Representative immunostaining results are shown for AID in the inflamed colonic mucosa from 3 UC cases. (original magnification, A–D, ×100).

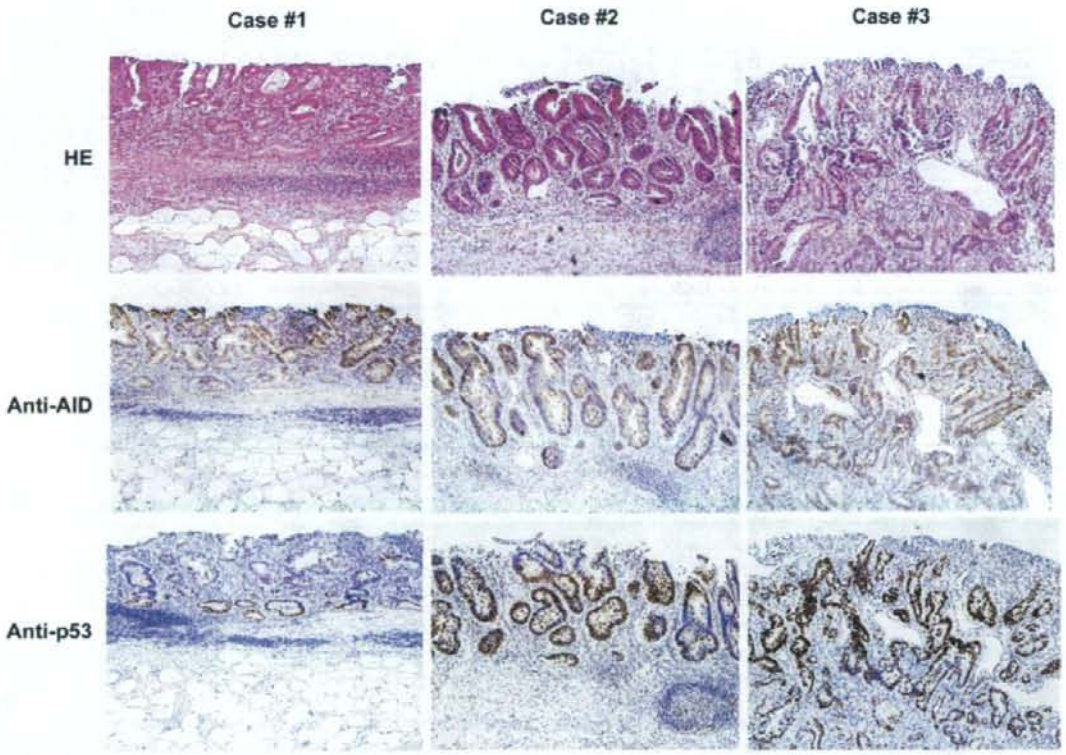

Figure 7. Expression of AID protein in colitis-associated neoplasma tissue specimens. Representative immunostaining for AID and TP53 protein is shown. Immunohistochemistry was performed on dysplasia (cases 1 and 2) or cancer (case 3) specimens from 3 patients with UC. (original magnification, 100×).

a well-known predisposing condition for colorectal cancer development.

Colonic mucosal inflammation is usually mediated by either an excessive Th1 T-cell response associated with increased IFN-γ and IL-12 secretion or an excessive Th2 T-cell response associated with increased IL-4, IL-5, and IL-13 secretion.[33,34] Although the concentration of the Th2 cell-driven cytokine IL-4 varies in UC colon tissue, UC is considered to have a Th2 profile.[33] Indeed, a recent study in mice suggests that production of IL-13 is an important pathologic factor for UC.[35] Moreover, UC has an atypical Th2 response, mediated by natural killer T cells that secrete IL-13,[36] and markedly elevated levels of IL-13 production are observed in UC patients.[29] In the current study, we demonstrated that 2 Th2 cytokines, IL-4 and IL-13, were capable of inducing endogenous AID expression in colonic epithelial cells. Interestingly, analysis using in animal models of colitis has shown that a predominance of Th2-type cytokines in inflamed colonic tissues, which mimics mucosal immunity in UC, enhances the development of colonic neoplasms.[37] Thus, we speculate that Th2 cytokine-mediated expression of AID in inflamed colonic epithelia might enhance the susceptibility to somatic mutations in tumor-related genes, leading to the formation of colonic neoplasms in patients with UC.

NF-κB transcription factors and the signaling pathways that activate NF-κB are central coordinators of inflammation-associated cancer development as well as immune responses.[38] NF-κB is activated in epithelial cells in the inflamed mucosa of patients with IBD.[24,39] Greten et al demonstrated that specific disruption of the IKK-β gene within enterocytes leads to a significant decrease in colitis-associated cancer multiplicity, suggesting that IKK-β-driven NF-κB contributes to the development of colitis-associated cancer.[40] It is thought that IKK-β-dependent NF-κB activation promotes the development of colorectal cancer via the transcriptional up-regulation of antiapoptotic target genes. In the present study, we identified AID as a target gene of the IKK-β-dependent NF-κB activation pathway in human colonic cells. Our findings demonstrate a novel link between the IKK-β-dependent NF-κB activation pathway and colitis-associated colorectal cancer development.

It is well established that several aspects of manifestation, including clinical features and genetic alteration characteristics, differ markedly between sporadic- and colitis-associated colorectal cancers.[5,6,41] Adenomatous polyps are considered to be the major precursor of sporadic colorectal cancers,[42] and inactivation of the *APC* gene is believed to be the initial event in sporadic colorectal cancer development, followed by changes in the *K-ras, DCC,* and *TP53* genes.[43] In contrast to sporadic cancer, an alteration in the *TP53* gene is usually an early event in the molecular pathogenesis of IBD-related colorectal cancer, whereas the mutation frequency of *APC* and *K-ras* is substantially lower than that in sporadic cancers. It is noteworthy that genetic alterations in the *TP53* gene likely precede dysplasia, the precancerous lesions in the inflammatory colon.[44] Moreover, there is an increased frequency of *TP53* mutations in noncancerous UC colon tissues.[45] The molecular mechanisms underlying the contribution of inflammatory conditions to the accumulation of *TP53* mutations are not well-defined. Here, we show that AID activation induced significant levels of *TP53* mutations in colonic mucosal cells. Thus, aberrant expression of AID might be involved in the generation of genetic alterations in the *TP53* gene in inflamed colonic epithelial cells.

An interesting point in our study is that the *APC, K-ras,* and *c-myc* genes were less frequently mutated by AID activation than the *TP53* gene in human colonic cells. These findings are consistent with previous observations that target gene selection of AID for somatic hypermutation varies among target cells.[8,9] Similar to its effect in colonic cells, aberrant AID expression in gastric epithelial cells induces nucleotide alterations in the *TP53* gene, whereas no mutations are induced in *c-myc*.[9] In contrast, AID expression in cultured hepatoma-derived cells results in the appearance of nucleotide alterations in the *c-myc* gene.[8] It is not clear why the *TP53* gene is more sensitive to AID activation in colonic epithelial cells. One possibility is that transcription levels of the genes targeted by AID are higher than other genes because AID-induced hypermutation depends on target gene transcription levels.[46,47] Consistent with this hypothesis, semiquantitative RT-PCR analysis revealed that *TP53* mRNA expression was higher than the *APC, K-ras,* and *c-myc* genes in LoVo cells. Further analysis is required to identify the specific target genes of AID-mediated mutagenesis in human colonic mucosal cells.

A previous study demonstrated that the development of hyperplasia of isolated lymphoid follicles in AID-deficient mice is associated with a 100-fold expansion of anaerobic flora in the small intestine.[48] On the other hand, oral administration of antigens from anaerobic bacterial flora of the intestine reduces the severity of experimental acute colitis.[49] Thus, further analyses are required to examine whether the AID expression level in the colon epithelium might influence the pattern of intestinal bacterial flora and the colonic inflammation in IBD patients.

In conclusion, we found that proinflammatory cytokines induced the aberrant expression of AID in human colonic cells, leading to the generation of somatic mutations in the host genome, including the *TP53* gene. Our findings provide a novel linkage between chronic inflammation and enhanced susceptibility to somatic mutations and an increased risk of colorectal cancers.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.06.091.

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