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Mechanisms of Ageing and Development 126 (2005) 732–739
mechanisms of ageing and development
www.elsevier.com/locate/mechagedev

Hematopoietic malignancies associated with increased *Stat5* and *Bcl-x_L* expressions in *Ink4a/Arf*-deficient mice

Young Hoon Sung [a,f,1], Junghwan Park [a,1], Bongkun Choi [a], Jaehong Kim [a], Cheolho Cheong [a], Yoon Sik Choi [a], Eun Young Yang [a], Minjae Lee [b], Jin Soo Han [c], Sang Chul Park [d], Tae-Hee Han [a], Tae Jin Kim [a], Jaewhan Song [e], Kunsoo Rhee [f], Han-Woong Lee [a,*]

[a] *Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, 300 Chonchon-Dong, Suwon 440-746, Republic of Korea*
[b] *Department of Veterinary Medicine, College of Animal Resources Science, Kangwon National University, Chunchon 200-701, South Korea*
[c] *Hae-Eun Biomedical Research Institute, Orient, Seoul, Republic of Korea*
[d] *Department of Biochemistry, Seoul National University College of Medicine, Seoul 110-799, Republic of Korea*
[e] *Department of Food Biotechnology, Sungkyunkwan University, Suwon 440-746, Republic of Korea*
[f] *School of Biological Sciences, Seoul National University, Seoul 151-742, Republic of Korea*

Available online 10 March 2005

Abstract

The *INK4a/ARF* locus, which encodes the two distinct proteins $p16^{INK4a}$ and $p14^{ARF}$, is frequently altered in various hematological malignancies as well as in other types of cancers in humans. In this study, we surveyed tumors that had spontaneously developed in *Ink4a/Arf*-deficient mice with an inbred FVB/NJ genetic background. We found that an *Ink4a/Arf*-deficiency exerted more severe effects on the induction of hematopoietic malignancies in mice with an inbred FVB/NJ genetic background than in mice with a mixed genetic background. We also provided the evidence that this prevalence of hematopoietic malignancies in *Ink4a/Arf*-deficient mice is associated with the upregulated expressions of *Stat5* and its transcriptional target, *Bcl-x_L*, both of which are involved in the regulation of hematopoiesis. These results suggest a possible implication of the *Ink4a/Arf* locus in the control of hematopoietic pathways by negatively regulating the Stat5-signalling pathways.

Keywords: $p16^{Ink4a}$; $p19^{Arf}$; Stat5; *Bcl-x_L*; Hematological malignancies

1. Introduction

The *Ink4a/Arf* locus encodes two tumor suppressors, $p16^{Ink4a}$ and $p14^{ARF}$ ($p19^{Arf}$ in mice) (Quelle et al., 1995), which regulate the Retinoblastoma protein (RB) and p53, respectively (Sherr, 2001). The direct association of $p16^{INK4a}$ with the cyclin D-dependent kinases, CDK4 and CDK6, inhibits their enzymatic activities, which maintains RB in its active, anti-proliferative state (Serrano et al., 1993). The interaction between $p19^{Arf}$ and Mdm2 prevents p53 degradation by simultaneously sequestering Mdm2 into the nucleolus and inhibiting its E3 ligase activity (Weber et al., 1999; Honda and Yasuda, 1999). The *INK4a/ARF* locus is localized on human chromosome 9p21, which has been known as one of the most frequently altered loci in many tumor types, including hematopoietic malignancies (Ortega et al., 2002; Malumbres and Barbacid, 2001). In order to study the significance of the mutations of the *INK4a/ARF* locus in humans several lines of murine models that are deficient in *$p16^{Ink4a}$*, *$p19^{Arf}$* or both have been developed (Serrano et al., 1996; Kamijo et al., 1997; Sharpless et al., 2001). All of these mutant mouse lines have manifested tumorigenic phenotypes, thus proving the role of this locus in tumorigenesis.

* Corresponding author. Tel.: +82 31 299 6135; fax: +82 31 299 6435.
E-mail addresses: syhdaum@hanmail.net (Y.H. Sung), hwl@med.skku.ac.kr (H.-W. Lee).
[1] These authors contributed equally to this work.

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doi:10.1016/j.mad.2005.01.007

Among the various roles of the *INK4a/ARF* locus, it has been highly suggested that this locus has important roles in regulating normal hematopoiesis (Ortega et al., 2002; Drexler, 1998; Krug et al., 2002). In humans, CD34+ hematopoietic progenitor cells show a high level of the $p16^{INK4A}$ expression, which is downregulated in later stages of hematopoiesis; thus, it has been suggested that it plays a role in the differentiation of these cells (Furukawa et al., 2000). In addition, the exogenous expression of $p16^{INK4A}$ in $p16^{INK4A}$-deficient hematopoietic cell lines induces both growth retardation and partial differentiation, which suggests that a $p16^{INK4A}$-deficiency may be responsible for their malignant phenotypes (reviewed in Krug et al., 2002). In humans, most mutations in the *INK4a/ARF* locus lead to the functional inactivation of both $p16^{INK4a}$ and $p14^{ARF}$ or preferentially of the $p16^{INK4a}$ function, even though there are rare mutations that seem to affect only the $p14^{ARF}$ function (Zhang and Xiong, 1999). Therefore, *Ink4a/Arf*-deficient mice, lacking both $p16^{Ink4a}$ and $p19^{Arf}$, are useful to delineate the effects of this deficiency on malignant hematopoiesis (Serrano et al., 1996). These mice have been predisposed to various spontaneous tumors including hematopoietic malignancies, such as B cell lymphomas in an early age, and are highly sensitive to carcinogenic treatments. It also has been shown that these mutant mice manifest abnormal extramedullary hematopoiesis (EMH), thus suggesting that this locus negatively regulates the cell-cycle in hematopoiesis. However, these studies were conducted using *Ink4a/Arf*-deficient mice with a heterogeneous genetic background. Since genetic background exerts profound effects on the spectrum of tumor development, e.g., p53-deficient mice (Donehower et al., 1995; Kuperwasser et al., 2000), the incidence of tumors observed in these *Ink4a/Arf*-deficient mice should be influenced by the heterogeneities from a mixed genetic background. In this regard, the analyses of spontaneous tumors in an inbred genetic background are expected to provide valuable information as to the role of an *Ink4a/Arf*-deficiency in tumorigenesis, and to clarify the involvement of the *Ink4a/Arf* locus in hematopoiesis.

In the present study, we surveyed spontaneous tumors arising in *Ink4a/Arf*-deficient mice with a FVB/NJ genetic background, and showed that an *Ink4a/Arf*-deficiency has more severe effects on the induction of hematopoietic malignancies such as lymphomas and histiocytic sarcomas in mice with this genetic background. In order to decipher the predominance of these malignancies at the molecular level, we conducted cDNA microarray experiment and observed that expressions of *Stat5* and its transcriptional target, *Bcl-x_L*, were upregulated in the spleen of tumor-free *Ink4a/Arf*-deficient mice. Interestingly they have been known to be involved in the regulation of both normal and malignant hematopoiesis (Calo et al., 2003; Socolovsky et al., 1999). Therefore, we provide the first evidence, which suggests that the *Ink4a/Arf* locus has important roles in regulating the Stat5-mediated signalling pathway, of which aberrations may have significant implications on the development of hematopoietic malignancies.

2. Materials and methods

2.1. Mice

The generation, initial characterization and genotyping of *Ink4a/Arf*-deficient mice were conducted as previously reported (Serrano et al., 1996). *Ink4a/Arf*-deficient mice with a C57BL/6, 129/Sv and SJL genetic background were backcrossed to FVB/NJ mice (10 generations). Fifty-seven *Ink4a/Arf*-deficient mice (30 females and 27 males) were monitored weekly over a 60-week period, and sacrificed when overt tumor development was detected, or when signs of morbidity were evident. In some cases, animals were monitored closely for several days and, if their condition worsened, they were sacrificed. All mice were housed in individually ventilated micro-isolation cages (Orient Co. Ltd., Seoul, Republic of Korea) in a specific pathogen-free (SPF) area of the Laboratory Animal Research Center of Sungkyunkwan University. This was fully accredited by AAALAC International for the animal care and use program.

2.2. Histological analyses

Tumors from nonautolyzed tissues were recovered from moribund mice. Tissue samples were fixed in 10% buffered formalin (Sigma). Osseous structures were decalcified with a hydrochloric acid solution.

Tissue samples were embedded in paraffin and were sectioned at 6 μm. For the histological examination, sections were stained with hematoxylin and eosin (H/E). Tumors were classified according to the criteria of Mohr (2001). For the immunohistochemical study, the rat anti-mouse F4/80 antibody was used as one marker for histiocyte detection.

2.3. Analysis of spleen cell populations by FACS

Cells (10^6) from spleen were washed in ice-cold PBS supplemented with 1% FBS and incubated with fluorophore-conjugated antibodies. The antibodies used in this experiment were as following: CD4-FITC, CD8-PE, IgM-FITC, and B220-PE (Pharmingen). After incubation of cells with antibodies at 4 °C for 1 h, cells were washed once and analyzed by FACS. Flow cytometry was performed on a FACScan (Becton Dickinson). Cell debris and background artifacts were electronically gated out, and the percentage of cells was computed using CellQuest software (Becton Dickinson).

2.4. cDNA microarray

Poly-A RNA was isolated from the spleens of *Ink4a/Arf*-deficient mice and littermates with the FastTrack™ 2.0

isolation kit (Invitrogen) according to the manufacture's instructions. Using these mRNAs, a complementary DNA (cDNA) microarray experiment was conducted (Incyte Genomics Inc., Palo Alto, CA).

2.5. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA was isolated from the spleens of an *Ink4a/Arf*-deficient mouse and wild type littermate using the TRIzol reagent (Invitrogen), and then it was incubated with RNase-free DNase I (Roche) for 30 min at 37 °C, followed by an additional incubation of 15 min at 70 °C. cDNA was generated from 8 μg of total RNA using the Superscript II RNase H-Reverse Transcriptase (Invitrogen). Primers were designed using the Primer Express software (Perkin-Elmer Biosystems). The primers used for the analysis were: 5′-GAAGCAGGCATCTGAGGACC-3′ and 5′-CGAAGGTGGAAGAGTGGGAGT-3′ for *Gapdh* (internal control); 5′-TGCAGTCCTGGTGTGAGAAG-3′ and 5′-AGATGATGTCCGTGATGGTG-3′ for *Stat5b*; and 5′-CGGAAGTGACCAGACACTGA-3′ and 5′-TAGGCCCAACCCTGTGATAG-3′ for *Bcl-x_L*. Reactions were performed in a total volume of 20 μl containing the SYBR Green PCR master mix (Perkin-Elmer Biosystems). Multiplex PCR was carried out in 96-well plate on a cDNA equivalent to 40 ng of the total RNA. Real-time PCR was performed using the GeneAmp PCR System 9600 (Perkin-Elmer Biosystems). Thermal cycling conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles at 95 °C for 30 s and 60 °C for 1 min. Data were collected using the ABI analytical thermal cycler. Each sample was amplified in triplicate.

2.6. Western blot analysis

Splenic tissues isolated from sacrificed mice were washed with PBS and lysed in lysis buffer [NP40 (1%), glycerol (10%), sodium fluoride (10 mM), β-glycerophosphate (50 mM), BSA (2 mg/ml), aprotinin (0.5%), pepstatin A (1 μM), and PMSF (0.2 mM)]. The protein concentration was assayed by the BCA kit (Pierce, Rockford, IL), and samples (50 μg) were subjected to 8 or 12% SDS-PAGE. Proteins were electrophoretically transferred to a nitrocellulose membrane (Hybond™ ECL, Amersham, Arlington Heights, IL) and the membranes were blocked in 20 mM Tris–HCl (pH 7.5) containing 250 mM NaCl, 0.05% Tween 20, and 5% non-fat dry milk. The membranes were incubated with a primary antibody (anti-Bcl-x_L and anti-Stat5a/b, Santa Cruz; anti-Stat1, anti-Stat3 and phosphorylation-specific anti-Stat5, cell signalling; anti-β-actin, Sigma). After they were washed with TBST (Tris–HCl [pH 7.5], 250 mM NaCl, 0.05% Tween 20), the membranes were incubated with a secondary antibody for 1 h, and visualized by the enhanced chemiluminescence procedure (ECL, Amersham).

3. Results

3.1. Distinct tumor incidence of Ink4a/Arf-deficient mice with a FVB/NJ genetic background

In order to carry out the detailed histopathological analysis of spontaneous tumors that develop in *Ink4a/Arf*-deficient mice with an inbred genetic background, we backcrossed *Ink4a/Arf-deficient* mice with a mixed genetic background to the FVB/NJ strain (10 generations), and surveyed tumor occurrences from 57 mutant mice (Table 1). Interestingly, the tumor spectrum of these mutant mice was different from that of mice with the mixed genetic background of C57BL/6, 129/Sv and SJL (Serrano et al., 1996).

Consistent with the previous study on a mixed genetic background, lymphomas were the most common tumors (33.8%, Table 1), and were frequently observed in mice between 21- and 30-week-old ages (54.5%, Table 2) (Serrano et al., 1996). Interestingly, more lymphomas were developed in female mice (45.9% versus 17.9%, Table 1). On the other hand, histiocytic sarcoma was the most frequent tumor type observed in male mice with the FVB/NJ genetic background (Table 1). In mice with a mixed genetic background, fibrosarcoma was the second major type of spontaneous tumor (Serrano et al., 1996).

Although other types of tumors, such as fibrosarcomas, hemangiosarcomas, alveolar/bronchiolar (A/B) adenoma and adenocarcinoma were also developed (Tables 1 and 2), our result suggests that an *Ink4a/Arf*-deficiency mainly elicits hematopoietic abnormalities in mice with a FVB/NJ genetic background. In conclusion, our data revealed that genetic background affects tumor susceptibility in *Ink4a/Arf*-deficient mice.

Table 1
The spectrum of spontaneous tumors in *Ink4a/Arf*-deficient mice of FVB/NJ genetic background

Type of tumor	No. of tumors (%)		
	Female[a]	Male[b]	Total
Lymphoma	17 (45.9)	5 (17.9)	22 (33.8)
Histiocytic sarcoma	12 (32.4)	9 (32.1)	21 (32.3)
Fibrosarcoma	1 (2.7)	6 (21.4)	7 (10.8)
Hemangiosarcoma	2 (5.4)	4 (14.3)	6 (9.2)
A/B tumor[c]	2 (5.4)	1 (3.6)	3 (4.6)
MFH[c]	0	2 (7.1)	2 (3.1)
Osteosarcoma	1 (2.7)	0	1 (1.5)
Mixosarcoma	0	1 (3.6)	1 (1.5)
Teratoma	1 (2.7)	0	1 (1.5)
Tubulostromal adenoma	1 (2.7)	0	1 (1.5)
	37 (100)	28 (100)	65 (100)

[a] $n = 30$. Twenty-five mice developed one tumor, 5 developed two independent tumors and 1 developed three independent tumors, yielding a total of 37 tumors.
[b] $n = 27$. Twenty-six developed one tumor and 1 developed two independent tumors, yielding a total of 28 tumors.
[c] A/B tumors, alveolar/bronchial tumors; MFH, malignant fibrous histiocytoma.

Table 2
Tumor incidence according to the age of *Ink4a/Arf*-deficient mice in FVB/NJ genetic background

No. of tumors (%)						
Type of tumor	Age (weeks)					
	<20	21–30	31–40	41–50	51–60	Total
Lymphoma	1 (1.5)	12 (18.5)	6 (9.2)	2 (3.1)	1 (1.5)	22 (33.8)
Histiocytic sarcoma	1 (1.5)	5 (7.7)	6 (9.2)	6 (9.2)	3 (4.6)	21 (32.3)
Fibrosarcoma	1 (1.5)	4 (6.2)	1 (1.5)	1 (1.5)	0	7 (10.8)
Hemangiosarcoma	1 (1.5)	2 (3.1)	2 (3.1)	1 (1.5)	0	6 (9.2)
A/B tumor[a]	0	3 (4.6)	0	0	0	3 (4.6)
MFH[b]	0	1 (1.5)	1 (1.5)	0	0	2 (3.1)
Osteosarcoma	0	0	0	0	1 (1.5)	1 (1.5)
Mixosarcoma	0	0	1 (1.5)	0	0	1 (1.5)
Teratoma	1 (1.5)	0	0	0	0	1 (1.5)
Tubulostromal adenoma	0	1 (1.5)	0	0	0	1 (1.5)
Total	5 (7.7)	28 (43.1)	17 (26.2)	10 (15.4)	5 (7.7)	65 (100)

[a] A/B tumors, alveolar/bronchial tumors; [b] MFH, malignant fibrous histiocytoma.

3.2. Malignant lymphomas in Ink4a/Arf-deficient mice with a FVB/NJ genetic background

In *Ink4a/Arf*-deficient mice with a FVB/NJ genetic background, the most affected organs were the spleen and liver, which exhibited hepatosplenomegaly (Fig. 1A). When histologically examined, the architecture of white and red pulps in the spleen was destroyed by the lymphoma cells (Fig. 1C). A microscopic inspection of the liver tissues showed the existence of periportal metastatic lymphoma cells (Fig. 1D). Also, lymphadenopathy, which contained proliferating lymphoblast cells, (Fig. 1B) was frequently observed (Fig. 1E). Since most lymphomas were generalized, they were found in the kidney, perirenal fat, adrenal gland, trachea, lungs and lymph nodes of submaxilla, axilla, mesentery, and ilia. Most of these tumors were classified as lymphoblastic, and a few lymphomas were leukemic, as determined by histopathology and a complete blood count (CBC). While the mature CD4+ or CD8+ T cell population was decreased (Fig. 2A and B), the population of B220+ and IgM+ B-lymphocytes was significantly increased in *Ink4a/Arf*-deficient mice diagnosed as lymphoma, indicating that this type of tumors may be B-cell lymphoma (Fig. 2C and D). Even though thymic lymphoma was not observed as previously reported (Serrano et al., 1996), the thymus was also affected with generalized lymphomas in nine cases that are likely originated from other tissues such as spleen. Together, these findings suggest that the *Ink4a/Arf* locus is an important factor for the maintenance of normal hematopoiesis, and its deficiency is associated with the appearance of malignant lymphomas in mice.

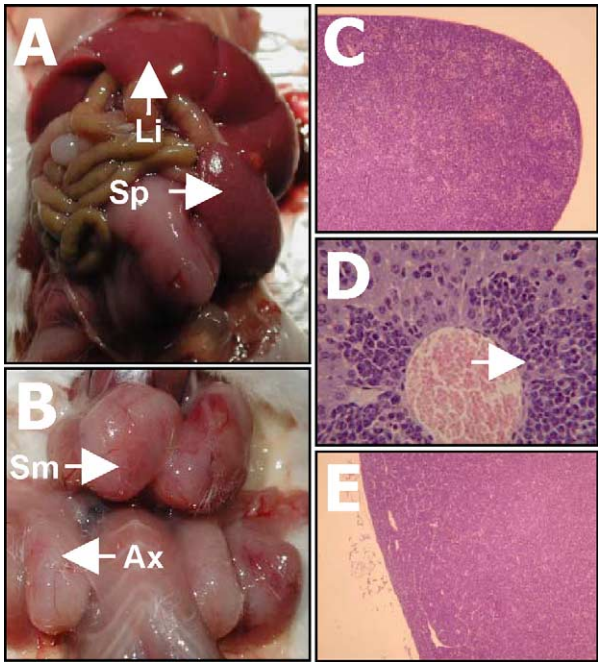

Fig. 1. Lymphomas spontaneously developed in *Ink4a/Arf*-deficient mice with a FVB/NJ genetic background. Gross appearance of an enlarged abdomen (A) and enlarged superficial lymph nodes (B). Histological examinations (H/E staining) revealing massively proliferating malignant lymphocytes throughout the white and red pulp in the spleen (C), periportal metastatic lymphoma cells in the liver (D), and proliferating lymphoblasts in the lymph node (E). Li, Liver; Sp, Spleen; Sm, submandibular lymph node; Ax, axillary lymph node.

3.3. Increased susceptibility to histiocytic sarcomas in Ink4a/Arf-deficient mice with a FVB/NJ genetic background

One of the striking features of *Ink4a/Arf*-deficient mice with a FVB/NJ genetic background was the prevalent incidence of histiocytic sarcomas (Table 1). These tumors were characterized by the presence of macrophages infiltrating the affected organs (Fig. 3). Although the liver (Fig. 3A and B) and spleen (Fig. 3C and D) were the most commonly affected organs with this type of tumor, it was occasionally observed in other tissues such as the lymph nodes (Fig. 3E and F), kidney (Fig. 3G and H), uterus and bone marrow (data not shown).

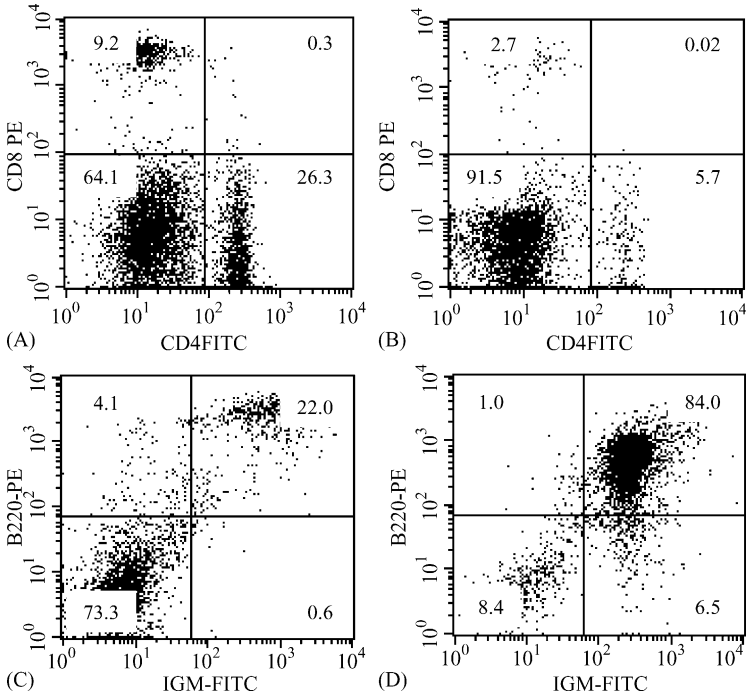

Fig. 2. Flow cytometric analysis of splenic cells. Splenic single-cell suspensions from spleen were obtained from *Ink4a/Arf*-deficient mice (B, D) and their wild-type siblings (A, C), and were immnunophenotyped for CD4 or CD8 positive T lymphocytes (A, B), and for IgM and B220 positive B lymphocytes (C, D). The results were computed from the acquisition of 10,000 events. The relative percentage of each subpopulation is indicated in the corresponding area of the diagrams.

In the liver, tumor cells occupied the sinusoids and surrounded the central vein with a granulomatous pattern, often eroding the walls and invading the lumen (Fig. 3B). Circumscribed nodular or multifocal infiltrates were observed by a microscopic examination. Neoplastic infiltrates consisted of large histiocytic cells with irregular basophilic nuclei, fibrillar, eosinophilic cytoplasm, and indistinct cytoplasmic outlines. We confirmed the presence of histiocytes using a marker (see Section 2; inset of Fig. 3B). The tumor was also marked by an enlargement of the spleen where tumor cells grew in the red pulp, forming packed nodular groups of neoplastic histiocytes as a uniform population (Fig. 3C and D).

In lymph nodes, the tumor cells, occasionally invading the surrounding adipose tissue, consisted of a prominent stromal component interspersed within a dense population of well-differentiated lymphocytes (Fig. 3F). Large nuclei and multinucleated giant cells were the common findings in these mice (inset of Fig. 3F). In the kidney, the tumor cells that floated within the peritoneum, invaded the cortex (Fig. 3H). These results showed that multiple organs or tissues were affected by histiocytic sarcomas in the *Ink4a/Arf*-deficient mice with a FVB/NJ genetic background.

3.4. Increased expressions of Stat5 and Bcl-x_L in the spleen of Ink4a/Arf-deficient mice

In order to decipher the molecular basis for the highly susceptible hematopoietic tumor development in *Ink4a/Arf*-deficient mice, we compared the gene expression profile of *Ink4a/Arf*-deficient mice with that of wild type mice (8-week-old prior to the onset of tumor development). At this age, there was no apparent abnormality in the cellular composition of *Ink4a/Arf*-deficient spleen, which was determined by flow cytometry using various cell surface markers such as CD45, CD3, CD4, CD8, B220, IgM, TER119, and GR-1 (data not shown). Using the mRNA isolated from the spleen, one of the most affected organs in the *Ink4a/Arf*-deficient mice (see above), a cDNA microarray experiment was conducted. We found that mice with the *Ink4a/Arf*-deficiency displayed altered expressions of various genes involved in signal transduction, transcriptional control, protein turnover, hematopoiesis, and cytoskeletons (data not shown). Among these genes, we took a special interest in the increased levels of *Stat5b* and *Bcl-x_L* transcription, based on their implication in normal and malignant hematopoiesis (Socolovsky et al., 1999; Bowman et al., 1999; Bromberg and Darnell, 2000; Sternberg and Gilliland, 2004). Thus, we further analyzed their upregulations in the spleens of the *Ink4a/Arf*-deficient mice.

Enhanced expressions of the *Stat5* and *Bcl-x_L* genes in splenic tissues of the *Ink4a/Arf*-deficient mice were confirmed in both the transcript and protein levels (Fig. 4). When assessed by real-time quantitative RT-PCR, relatively higher levels of *Stat5b* (2.5-fold increase) and *Bcl-x_L* (3.2-fold increase) mRNAs were verified in the spleens of the *Ink4a/Arf*-deficient mice (Fig. 4A and B). Consistently, the Western blot analyses showed the

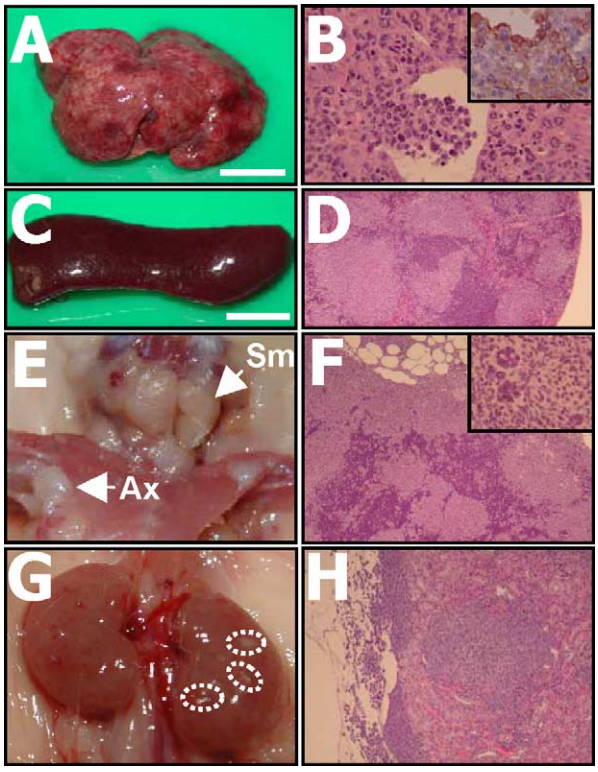

Fig. 3. Representative histiocytic sarcomas of *Ink4a/Arf*-deficient FVB/NJ mice. Gross appearance of the liver with an irregular surface (A), spleen showing splenomegaly (C), enlarged submandibular (Sm) and axillary (Ax) lymph nodes (E), and kidney showing pale red color with white spotted lesions (white dotted areas) (G). Histological examinations (H/E staining) of the liver (B; inset, immunohistochemical detection of macrophages using the cell surface marker F4/80), spleen (D), submandibular lymph node (F; inset, a photograph with high magnification), and kidney (H). Scale bars in A and C: 1 cm.

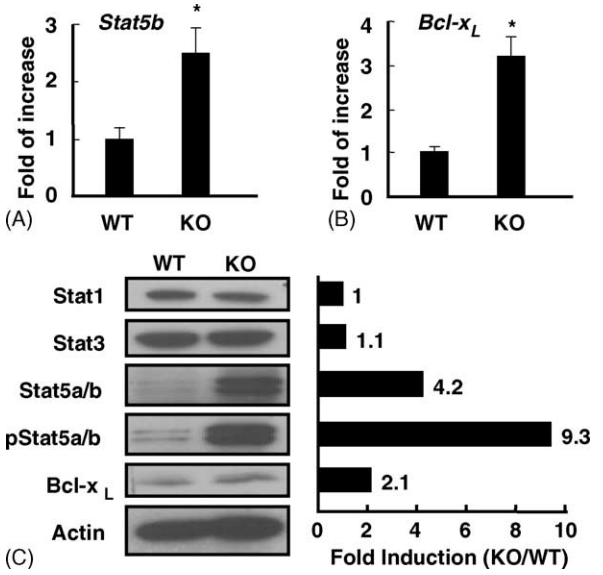

Fig. 4. Examination of *Stat5* and *Bcl-x_L* expressions in the spleen of *Ink4a/Arf*-deficient mice. Prior to tumor development, RNAs and proteins were prepared from the spleens of wild type and *Ink4a/Arf*-deficient mice. Real-time RT-PCR analysis showing the relative levels of *Stat5b* (A) and *Bcl-x_L* (B) mRNAs. * $p < 0.005$, wild type vs. *Ink4a/Arf*-deficient mice, Student's test. Western blot analyses (C) of Stat proteins and *Bcl-x_L* (pStat5a/b; phosphorylated Stat5a/b proteins). The graph indicates the relative expressions in *Ink4a/Arf*-deficient (KO) mice to wild type (WT) mice, which are normalized with actin signals as the loading control.

increased protein levels of Stat5 (4.2-fold increase) and *Bcl-x_L* (2.1-fold increase) (Fig. 4C). The deficiency of the *Ink4a/Arf* locus, however, did not alter the protein levels of the other Stat family members, such as Stat1 and Stat3 (Fig. 4C). Since the transcriptional activity of Stat proteins is regulated by their phosphorylation status (Darnell, 1997), we also examined the activation status of Stat5 proteins with a phosphorylation-specific antibody against Stat5. As shown in Fig. 4C, the phosphorylation level of Stat5 protein was significantly increased (9.3-fold). Although the activation of Stat5 can be achieved by erythropoietin receptor (Epo^R) (Socolovsky et al., 1999), the level of Epo^R was not significantly altered, which indicates that this activation of Stat5 is independent of erythropoietin (Epo) in the spleen of *Ink4a/Arf*-deficient mice (data not shown).

4. Discussion

This study demonstrated that genetic background plays an important role in determining the phenotypic expression of an *Ink4a/Arf*-deficiency. Our results suggest that the genetic environments or modifier genes in the FVB/NJ strain may be responsible for the enhanced development of histiocytic sarcomas in *Ink4a/Arf*-deficient mice. Although a few studies have already described similar observations in this genetic background (Artandi et al., 2002; Sharpless et al., 2004), our results have raised an additional possibility that sexual difference may affect the incidence of specific tumors such as lymphomas and histiocytic sarcomas in *Ink4a/Arf*-deficient mice (Table 1). Furthermore, our data may also indicate why different individuals with the same germ line mutation in the *INK4a/ARF* locus display variations in the susceptibility to tumor development and in the tumor spectrum.

The *Ink4a/Arf* locus has an influence on growth and self-renewal kinetics of hematopoietic progenitor cells (Lewis et al., 2001). When cultured in vitro, those cells of *Ink4a/Arf*-deficienct mice show an increased longevity and immortality, which suggests that this locus intrinsically regulates the fates of hematopoietic progenitor cells. Several pathways can be employed to explain the mechanistic involvement of the *Ink4a/Arf* locus in the development of hematopoietic malignancy. Since $p16^{Ink4a}$ indirectly maintains Rb in its active, anti-proliferative state (Serrano et al., 1993), its loss, thereby disabling the "Rb pathway", will potentiate hematopoietic progenitor cells to progress into a cell-cycle rather than to differentiate into specific cell lineages. Indeed, it has been well known that a germ line mutation of *Rb* results in hematopoietic defects in

developing mouse embryos (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Furthermore, the compound mutant mice of *E2F1* and *E2F2* reveal profound defects in the hematopoietic development of multiple cell lineages, showing that a decrease in cellularity is associated with an impeded S phase progression in its progenitor cells (Li et al., 2003). This line of evidence emphasizes importance of the Rb pathway, which is regulated by the *Ink4a/Arf* locus in normal hematopoiesis.

On the other hand, the loss of $p19^{Arf}$ disrupts signalling through the $p19^{Arf}$-Mdm2-p53 pathway (Weber et al., 1999; Honda and Yasuda, 1999). It is evident that the repression of $p19^{Arf}$ is associated with leukemias in humans harboring intact p53 (Linggi et al., 2002). This means that the lack of $p19^{Arf}$ impedes the normal function of p53, which is associated with cell-cycle control, apoptosis, and the maintenance of genomic integrity, thereby causing malignancies in hematopoietic system as well as in other organs or tissues.

However, our data suggest that an additional mechanism might be accompanied with the deficiency of the *Ink4a/Arf* locus in mice. We observed the upregulations of *Stat5* and its downstream, *Bcl-x_L*, which implies that the signalling pathway through Stat5 is strengthened in *Ink4a/Arf*-deficient mice (Fig. 4). Stat family members such as Stat1, Stat3, and Stat5 participate in diverse biological processes, and play an important role in controlling cell-cycle progression and apoptosis, and thus contribute to oncogenesis (Calo et al., 2003). Our data point out that rather than Stat1 and Stat3, it is Stat5 that might be involved in the tumorigenesis of *Ink4a/Arf*-deficient mice. While Stat5 is activated upon Epo^R stimulation in erythropoiesis, its activation in *Ink4a/Arf*-deficient mice appears to be independent of Epo^R stimulation, which indicates the existence of other regulatory pathways (Socolovsky et al., 1999).

The activation of Stat5 is accompanied in the progression of diverse hematopoietic malignancies (Sternberg and Gilliland, 2004). A recent report showed that the overexpression of Stat5a or Stat5b within the lymphoid compartment induces the development of T cell lymphoblastic lymphomas in transgenic mice (Kelly et al., 2003). Our data indicate that the *Ink4a/Arf* locus may be located upstream of the Stat5 signalling pathway, which has been known to have significant roles in regulating normal and malignant hematopoiesis.

What is the possible molecular basis for the upregulation of Stat5 and its downstream, *Bcl-x_L*, in *Ink4a/Arf*-deficienct mice? The ectopic expression of $p16^{INK4a}$ induces growth retardation and partial differentiation in an Rb-dependent manner (reviewed in Krug et al., 2002). Moreover, this expression also downregulates the expression of *Bcl-x_L*, concomitant with the partial differentiation and apoptosis in cells of an erythroid lineage (Minami et al., 2003). Considering the transcriptional regulation of *Bcl-x_L* by Stat5, it is plausible that Stat5 might also be regulated by $p16^{INK4a}$. However, we still cannot rule out the possible involvement of $p19^{Arf}$ since p53 can exert a significant effect on IL-6-induced Stat5-masking (Rayanade et al., 1998). Unravelling the more detailed linkage between the germ line mutation of *Ink4a/Arf* locus and aberrant Stat5 activation will be the main issue of the future study, and has an enormous potential to provide us with the clues to understand the roles of *Ink4a/Arf* locus in vivo.

Although more analysis should be conducted for further studies, we have provided the first evidence that suggests the *Ink4a/Arf* locus negatively regulates the Stat5 signalling pathway, which has significant implications in the normal control of hematopoiesis. The results from this study enlighten us on a novel aspect of tumorigenesis associated with an *INK4a/Arf*-deficiency, and will thus provide therapeutic implications of *STAT5* as a molecular target for the *INK4a/ARF*-deficient tumors in humans.

Acknowledgements

This work was supported by the grants from the Ministry of Health and Welfare (02-PJ1-PG3-21001-0007), 21C Frontier Functional Human Genome Project (FG01-0304-002-1-0-0) and BRC-frontier (M103KV010018-03K2201-01830) from the Ministry of Science and Technology, Korea Science and Engineering Foundation through the Science Research Center–Molecular Therapy Research Center, and Korea Research Foundation (015-C00398).

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