"BALB/c mice obtained commercially were found to differ significantly from the standard phenotype of BALB/c strain mice. Isoenzyme tests and H-2 haplotype analyses indicated that the majority of mice from two of the three sources tested appeared mixed, frequently heterozygous, and did not consistently express either the expected H-2 or glucose phosphate isomerase type."

This fact was introduced as a big problem in the journal *Science*.[4]

We reported that some NZB substrains in Japan were genetically contaminated as shown in Table 11.1. Five of 26 substrains showed different alleles from those of the standard NZB strain at three loci in #503 and #401, and at four loci in #302, #305, and #311. These were NZB substrains derived from the original strain (#302) at different times. These allele differences could be brought about by genetic contamination with other strains. Recently, genetic contamination in 129 substrains was also reported.[5–7]

One of the serious problems for maintaining ordinary inbred strains is the genetic contamination described above. The genetic homogeneity of inbred strains will be lost, and animal experiments with contaminated strains will result in the complications described below.

SPONTANEOUS MUTANT AND GENETICALLY ENGINEERED MOUSE PHENOTYPES VARY WITH GENETIC BACKGROUND

It was reported that phenotypes, especially disease-related ones, change on different genetic backgrounds when incipient and true congenic strains are created. Sundberg et al.[8] reported that the flaky skin phenotypes with seven parameters (acanthosis, hypergranulosis, orthokeratosis, parakeratosis, intracorneal microabscess, dilated dermal capillaries, and dermal inflammation) varied on different genetic backgrounds as shown in Table 11.2. We observed that a second allelic mutation (*fsn*^{Jic}) of the flaky skin locus showed different phenotypes when this mutant allele was transferred onto BALB/cByJcl, C3H/HeJJcl, C57BL/6JJcl, and DBA/2JJcl by back-crossing (Katoh, unpublished data).

According to TBASE[9] the number of genetically engineered strains of targeted mutant mice and transgenics in 2003 were 1103 and 758, respectively.

With the development of genetically engineered mice, we face different problems from those seen in the ordinary inbred strains. One is the heterogeneity of genetic backgrounds and the other is the change in phenotypes on different genetic backgrounds. The author has surveyed genetically engineered mouse strains listed in the JAX catalogue.[10] About 20% of 246 transgenics and about 30% of 511 targeted mutant stocks were back-crossed to create congenic strains. Therefore, they were noninbred at that time and heterogeneous at many loci differing between donor and recipient strains.

Thus, a considerable number of genetically engineered mice will be in the same genetic situations as genetically contaminated mice. There is no doubt

TABLE 11.1
Typical Genetic Contamination Observed in NZB Substrains in Japan

	Chromosome No. and gene loci*																	
	1	1	1	2	3	4	4	5	6	7	7	8	8	9	9	9	11	17	17
Substrain	*Idh1*	*Pep3*	*Akp1*	*Hc*	*Car2*	*Mup1*	*Gpd1*	*Pgm1*	*Ldr1*	*Gpi1*	*Hbb*	*Es1*	*Es2*	*Thy1*	*Mod1*	*Trf*	*Es3*	*H2K*	*H2D*
Control	*a*	*c*	*b*	*0*	*a*	*a*	*b*	*b*	*a*	*a*	*d*	*b*	*b*	*b*	*b*	*b*	*c*	*d*	*d*
#302	*a*	*c*	*b*	***1***	*a*	***b***	*b*	*b*	*a*	*a*	***s***	***a***	*b*	*b*	*b*	*b*	*c*	*d*	*d*
#305	*a*	*c*	*b*	***1***	*a*	***b***	*b*	*b*	*a*	*a*	***s***	***a***	*b*	*b*	*b*	*b*	*c*	*d*	*d*
#311	*a*	*c*	*b*	***1***	*a*	***b***	*b*	*b*	*a*	*a*	***s***	***a***	*b*	*b*	***b***	*b*	*c*	*d*	*d*
#401	*a*	*c*	***a***	*0*	*a*	***b***	*b*	*b*	*a*	*a*	*d*	***a***	*b*	*b*	*b*	*b*	*c*	*d*	*d*
#503	*a*	***b***	***a***	*0*	*a*	*a*	*b*	*b*	*a*	*a*	*d*	***a***	*b*	*b*	*b*	*b*	*c*	*d*	*d*

*See Table 11.3. Allele shown in bold differs from that of the control strain.

TABLE 11.2
Flaky Skin (*fsn*^{l}) Phenotypes Modified by Genetic Backgrounds[a]

Strain	Acanthosis	Hyper-granulosis	Ortho-keratosis	Para-keratosis	Intra-corneal Micro-abscess	Dilated Dermal Capillaries	Dermal inflam-mation
A/J	S	Mi	S	F	F	Y	Mo-S
BALB/cByJ (N7)	Mo	S	Mo	R	R	Y	Mi-Mo
A/J × BALB/cByJ	Mo-S	Mi	S	F	F	Y	Mo-S
C57BL/6J (N6)	S	Mo	S	F	F	Y	Mo

[a] S: severe; Mo: moderate; Mi: mild; F: focal; R: rare; Y: yes.
Source: Modified data of Sundberg.[8]

that if we desire reasonable and stable results in animal experimentation, we have to develop and maintain inbred and congenic strains with appropriate genetic quality control.

GENETIC MONITORING (GENETIC QUALITY TESTING) OF LIVE MICE

Genetic monitoring systems were developed to ensure genetic quality of experimental animals at the Central Institute for Experimental Animals (Kawasaki, Japan) designated as the ICLAS Monitoring Center by ICLAS (International Council for Laboratory Animal Science) in 1979.[11] Since then, we have been responsible for developing the genetic monitoring system for live animals as follows.

Markers for Genetic Monitoring

There are various types of genetic markers that can be used for genetic testing. They are divided into six groups according to their biological functions, phenotypes, and detecting methods as follows: (1) biochemical markers (*Hbb*, *Gpi1*, etc.), (2) immunological markers (*H2*, *Thy1*, etc.), (3) morphological markers (coat colors, etc.), (4) cytogenetic markers (C bands, etc.), (5) molecular genetic markers (minisatellites, microsatellites, etc.), and (6) pharmacogenetic markers (alcohol preference, etc.). Well-known inbred strains such as C57BL/6J and DBA/2J have been characterized for these markers. Almost all data are available in a mouse genetic data book[12] or online at Mouse Genome Informatics.[13]

When markers for genetic quality testing are selected from these six groups, the following conditions should be considered: (1) Exact (clearly detected using easier techniques), (2) Easy (quickly detected using easier techniques), (3) Efficient

(polymorphic among inbred strains), and (4) Economic (detected using cheaper materials and equipment). We call these specific requirements the 4Es by taking the first letter from each category. We concluded that biochemical and immunological markers were among the six types of markers that were satisfactory for meeting the goals of the 4Es. The ICLAS Monitoring Center selected 28 loci (19 biochemical and 9 immunological markers) for mice as shown in Table 11.3.

Recently, microsatellite DNA markers detected using PCR (polymerase chain reaction) were used for gene mapping with linkage analyses. We attempted to use microsatellite markers for genetic quality testing of cell lines derived from experimental animals. When we selected microsatellite markers as markers for genetic quality testing, we paid special attention to the first E (Exact) of the 4Es, because it was demonstrated that not all microsatellite DNA markers show genetic stability of their PCR products (Katoh, unpublished data). After characterizing many microsatellite markers on every chromosome in the mouse, we have finally selected 20 microsatellite markers showing genetic stability (data not shown).

Sets of Markers for Genetic Quality Testing

Sets of markers for genetic quality testing are classified into three profiles according to their purposes.

1. *Genetic Profile:* Many markers are investigated for each strain and the genetic differences from the standard strain are clarified. The genetic profile of an inbred strain consists of genotypes of the markers listed in Table 11.3 and certifies genetic condition (genetic quality) of a given mouse strain. Other biochemical and immunological markers selected on the basis of the requirements for genetic quality testing may be added on demand. There is no limitation with respect to the number of markers, but the markers shown in Table 11.3 will be adequate for the purpose mentioned above.
2. *Monitoring Profile:* A monitoring profile is prepared using a set of markers selected on the basis of genetic quality testing. The ICLAS Monitoring Center selected 19 markers in mice as shown in Table 11.3. The monitoring profile of the strain certifies genetic condition (genotypes) of the strain (Table 11.4).
3. *Critical Subset Profile*: A critical subset profile is prepared using a set of restricted markers selected to identify a limited one among inbred strains maintained or produced in a room or a facility. As shown in Figure 11.1, six typical albino mouse strains can be differentiated using a critical subset consisting of only four biochemical markers. They are enough to discriminate one from another. If genetic contamination occurs between them, it is easy to expose it using a critical subset.

TABLE 11.3
Genetic Markers for Genetic Quality Testing in Mice

Chromosome No.	Gene Symbol and Gene Name (Old Symbol and gene name in parenthesis)		Genetic Profile	Monitoring Profile
		Biochemical markers (19 loci)		
1	*Idh1*	Isocitrate dehydrogenase-1	×	×
1	*Pep3*	Peptidase-3	×	×
1	*Akp1*	Alkaline phosphatase-1	×	×
3	*Car2*	Carbonic anhydrase-2	×	×
4	*Mup1*	Major urinary protein-1	×	×
4	*Gpd1*	Glycerol-3-phosphate dehydrogenase 1 (soluble)	×	×
5	*Pgm1*	Phosphoglucomutase-1	×	×
6	*Ldr1*	Lactate dehydrogenase regulator-1	×	×
7	*Gpi1*	Glucose phosphate isomerase-1	×	×
7	*Hbb*	Hemoglobin beta chain complex	×	×
8	*Es1*	Esterase-1	×	×
8	*Es2*	Esterase-2	×	×
9	*Mod1*	Malic enzyme, supernatant	×	×
9	*Trf*	Transferrin	×	×
11	*Hba*	Hemoglobin alpha chain complex	×	
11	*Es3*	Esterase-3	×	×
14	*EsD (Es10)*	Esterase D/formylglutathione hydrolase (Esterase-10)	×	
14	*Nptx1 (Np1)*	Neuronal pentraxin (Nucleoside phosphorylase-1)	×	
17	*Glo1*	Glyoxalase-1	×	
		Immunological markers (9 loci)		
2	*Hc*	Hemolytic component (synonyms:C5)	×	×
6	*Cd8a (Ly-2)*	Antigen, alpha chain (Lymphocyte antigen 2)	×	
6	*Cd8b1 (Ly-3)*	Antigen, beta chain (Lymphocyte antigen 3)	×	
9	*Thy1*	Thymus cell antigen 1, theta	×	×
12	*IghC*	Immunoglobulin heavy chain constant region	×	
17	*H2K*	Histocompatibility 2, K region	×	×
17	*H2D*	Histocompatibility 2, D region	×	×
17	*C3*	Complement component-3	×	
19	*Cd5 (Ly1)*	CD5 antigen (Lymphocyte antigen 1)	×	

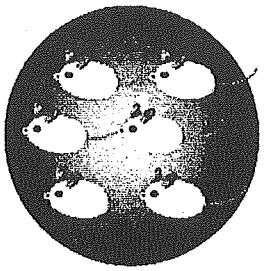
Six albino mice	Test markers				Strain
	*Idh*1 ⇒	*Hbb* ⇒	*Es*3	⇒ *Mod*1	
	*a*	*d*	*c*	nd	A
	a	*d*	*a*	nd	BALB/c
	a	*s*	*c*	nd	DDD/1
	b	*d*	nd	*b*	AKR
	b	*d*	nd	*a*	NZW
	b	*s*	nd	*a*	SJL.

FIGURE 11.1 A critical subset for strain identification among six albino mice; *a–d* and *s* are alleles at tested genes, *Idh1*, *Hbb*, *Es3*, and *Mod1*. nd: not done.

Categories of Genetic Quality Testing

Depending on different purposes, genetic tests are divided into three categories as follows.

1. *Characterization:* This is carried out to study or demonstrate genotypes of inbred strains and also to define genetic profiles of newly bred strains. This test is performed once when strains are newly introduced or established.
2. *Genetic Monitoring I:* This is periodically carried out to confirm the monitoring profile of a given inbred strain in order to demonstrate that genetic contamination or mistakes do not occur in the strain. Testing is performed on a scheduled basis to determine if genetic quality of the strain has been maintained.
3. *Genetic Monitoring II:* This is carried out in order to determine whether genetic contamination or mistakes occurred. Several markers showing genetic polymorphisms among the strains are selected as the test items from monitoring markers (Table 11.3).

Schedule of Genetic Monitoring

Test Frequency

We recommend that in the case of strain maintenance and long-term production, three kinds of tests be performed in accordance with the following schedule.

Scheduled testing is recommended in the case of strain maintenance and long-term production, but spot testing can be used in short-term cases or when the colony is small.

Immediately after introduction of new strains, these strains are characterized to confirm genotypes of the strain. These characteristics are monitored every several years after introduction. If the introduced strain is correct, the strain is monitored periodically (once a year) at prescribed schedules (see below).

When individuals with different coat colors appear or the litter sizes sharply increase in inbred strains, there is a possibility of genetic contamination. At this point a critical subset test should be performed immediately.

Monitoring Schedule

In the case of strain maintenance populations, a pair of female and male at F(n) generation surviving the next generation F(n + 1) within the line or six mice from the F(n + 1) generation are selected for testing. In the case of production populations, six animals are selected at random from the population. In the case of closed colonies, 50 mice are selected at random from the populations. See Table 11.5.

GENETIC MONITORING FOR CELL LINES, GAMETES, AND EMBRYOS

Genetic quality of *in vitro* cultured cells derived from experimental animals as well as live animals should be checked, because the genetic quality can be destroyed by the following factors: mixing different cell lines, mishandling, and natural changes occurring on chromosomes and DNA. The first and the second problems can be prevented and identified using genetic techniques. The third factor is inevitable. These errors will be causes of producing genetically changed (contaminated) cell lines and spreading them to other facilities. When genetic monitoring is applied to *in vitro* cultured cell lines, we should pay special attention to the species identification as well as the strain identification. Some of the methods used for this purpose are: (1) karyotyping (chromosome analysis), (2) isozyme analysis using electrophoresis, (3) DNA fingerprinting (minisatellite DNA), and (4) chromosome painting using specific DNA probes. Although these approaches are useful for identification of the animal species, the techniques and procedures are specific and complicated.

Alternatively, genetic resource banks preserving gametes (sperm and ova) and the early stage of embryos (two-cell, eight-cell embryos, etc.) were recently established and are now used worldwide. Repositories have responsibilities to produce and distribute transgenic mice, targeted mutant mice, and strains carrying various mutations. Even in embryo and sperm banks, one should always consider mishandling of straws or cryotubes containing sperm and embryos, because mishandling will lead to mixes between different strains which will result in genetic contamination.

We proposed that cells, gametes, and early-stage embryos should be identified by: species, sex, and strain. PCR (polymerase chain reaction) has been developed

TABLE 11.4
Monitoring Profiles of the Typical Inbred Strains of the Mouse

	Chromosome No. & Gene loci																		
	1	1	1	2	3	4	4	5	6	7	7	8	8	9	9	9	11	17	17
Strains	*Idh1*	*Pep3*	*Akp1*	*Hc*	*Car2*	*Mup1*	*Gpd1*	*Pgm1*	*Ldr1*	*Gpi1*	*Hbb*	*Es1*	*Es2*	*Thy1*	*Mod1*	*Trf*	*Es3*	*H2K*	*H2D*
A	*a*	*b*	*b*	*0*	*b*	*a*	*b*	*a*	*a*	*a*	*d*	*b*	*b*	*b*	*a*	*b*	*c*	*k*	*d*
AKR	*b*	*b*	*b*	*0*	*a*	*a*	*b*	*a*	*a*	*a*	*d*	*b*	*b*	*a*	*b*	*b*	*c*	*k*	*k*
BALB/c	*a*	*a*	*b*	*1*	*b*	*a*	*b*	*a*	*a*	*a*	*d*	*b*	*b*	*b*	*a*	*b*	*a*	*d*	*d*
CBA/J	*b*	*b*	*a*	*1*	*b*	*a*	*b*	*a*	*a*	*b*	*d*	*b*	*b*	*b*	*b*	*a*	*c*	*k*	*k*
CBA/N	*b*	*b*	*b*	*1*	*a*	*a*	*b*	*b*	*b*	*b*	*d*	*b*	*b*	*b*	*b*	*a*	*c*	*k*	*k*
C3H/He	*a*	*b*	*b*	*1*	*b*	*a*	*b*	*b*	*a*	*b*	*d*	*b*	*b*	*b*	*a*	*b*	*c*	*k*	*k*
C57BL/6	*a*	*a*	*a*	*1*	*a*	*b*	*a*	*a*	*a*	*b*	*s*	*a*	*b*	*b*	*b*	*b*	*a*	*b*	*b*
DBA/1	*b*	*b*	*a*	*1*	*a*	*a*	*a*	*b*	*a*	*a*	*d*	*b*	*b*	*b*	*a*	*b*	*c*	*q*	*q*
DBA/2	*b*	*b*	*a*	*0*	*b*	*a*	*b*	*b*	*a*	*a*	*d*	*b*	*b*	*b*	*a*	*b*	*c*	*d*	*d*
KK	*a*	*b*	*b*	*0*	*a*	*b*	*a*	*a*	*a*	*b*	*d*	*b*	*a*	*b*	*a*	*b*	*c*	*b*	*b*
NZB	*a*	*c*	*b*	*0*	*a*	*a*	*b*	*b*	*a*	*a*	*d*	*b*	*b*	*b*	*b*	*b*	*c*	*d*	*d*

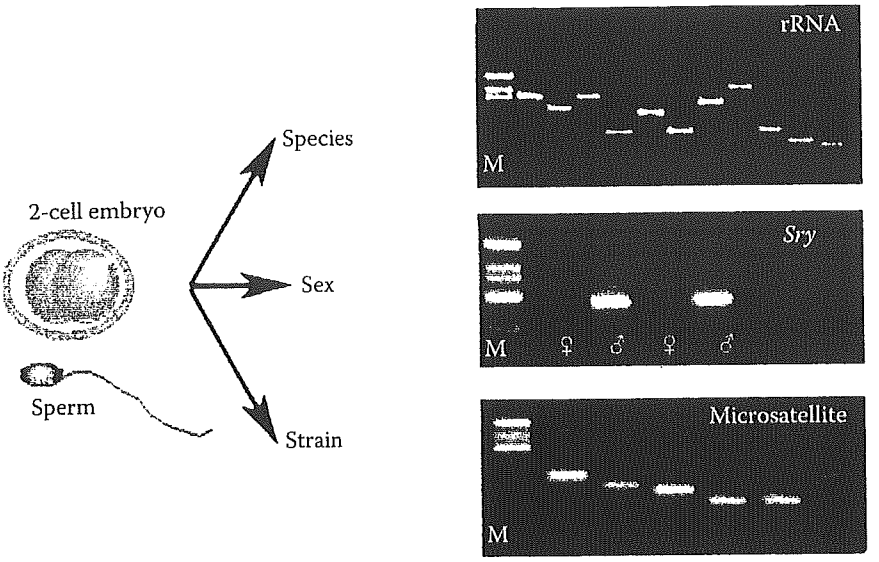


FIGURE 11.2 Three classifications (species, sex, and strain) for identification of embryos and sperm; M: DNA size marker; *rRNA*: ribosomal RNA gene; *Sry*: sex determining region of Y chromosome.

to amplify a part of the desired gene (DNA sequence). Using PCR, microsatellite DNA markers dispersed on the mouse genome, which are detected as simple sequence length polymorphisms (SSLPs), were developed to study linkage of genes, especially disease-related genes in mice.

Species Identification

Naito et al.[14] developed a method discriminating animal species with a ribosomal ribonucleic acid gene (*rRNA*). They designed and synthesized a pair of primers to detect a variable region of DNA coding 28S rRNA. They successfully identified various animal species with PCR products of their rRNA as shown in Figure 11.2. We confirmed that this method could be applied to identify the animal species of the cell lines of rats.[15]

Sex Identification

To identify sexes of cell lines, three pairs of primers for detecting DNA sequences of the Y chromosome of mice and rats, respectively, were synthesized according to the literature. As shown in Figure 11.2, mice and rat females were discriminated from males by not observing Y-specific PCR products in agarose gel.

Strain Identification

We performed a preliminary study to select the most effective microsatellite markers that identify as many mouse strains as possible (see Table 11.6). We

TABLE 11.5
Monitoring Schedule (Example)[a]

Period (year)	1	2	3	4	5
Type of test	C	MI	MII	MI	MII
No. animals	2	2 or 6	2 or 6	2 or 6	2 or 6

[a] C: Characterization; MI: Monitoring I; MII: Monitoring II.

TABLE 11.6
Monitoring Profiles of the Typical Inbred Strains of the Mouse

Strain	Origin	*D3Mit54*	*D5Mit18*	*D6Mit15*	*D8Mit50*
1. C57BL/6	Jic	1	1	1	4
2. MSM	Ms	1	1	1	5
3. NC/Nga	Jic	1	1	3	3
4. MRL/MpJ-*lpr*	Jic	1	2	1	1
5. BALB/cA	Jic	1	2	3	2
6. IQI	Jic	1	2	3	3
7. C3H/HeN	Crj	1	2	3	4
8. SWR/J	Ms	1	2	4	1
9. NFS/N	Jic	1	2	4	3
10. NOD/Shi-*scid*	Jic	1	2	4	3
11. NZB	Crj	2	1	1	2
12. AKR/J	Jic	2	2	1	2
13. NZW	Crj	2	2	1	2
14. 129/J	Ms	2	2	3	2
15. DBA/2J	Jic	2	2	3	3

selected markers that are detected with the same PCR condition. We selected four microsatellite markers, *D3Mit54*, *D5Mit18*, *D6Mit15*, and *D8Mit50*. If we have four markers with two alleles, they are theoretically enough to discriminate 16 strains. As shown in Table 11.6, 15 typical inbred strains were divided into 13 groups. Therefore, genetic monitoring should be applied at appropriate stages of embryos and gametes to confirm genetic accuracy of the strains.

Genetic monitoring of embryos and gametes are performed using genomic DNA distributed in all tissues and organs. As shown in Figure 11.3, we successfully performed genetic quality testing of embryos using eight-cell stage embryos.[16]

Genotyping of Transgenes and Targeted Genes

We introduced the fluorescence *in situ* hybridization (FISH) technique for chromosomal mapping of transgenes. Figure 11.4 is an example of chromosomal mapping

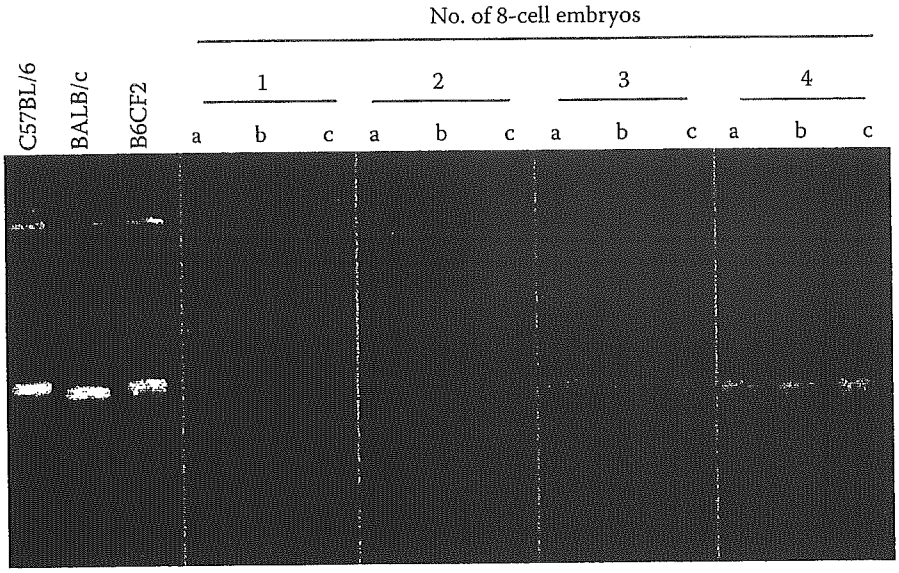


FIGURE 11.3 Genotyping of the MIT marker (*D5Mit18*) performed using eight-cell embryos. A method of preparation of template DNA for PCR was established using the lysis buffer (1 × PCR reaction buffer supplemented with proteinase K at a concentration of 40 μg/ml). Tests were performed three times; a, b, and c are experiment numbers.

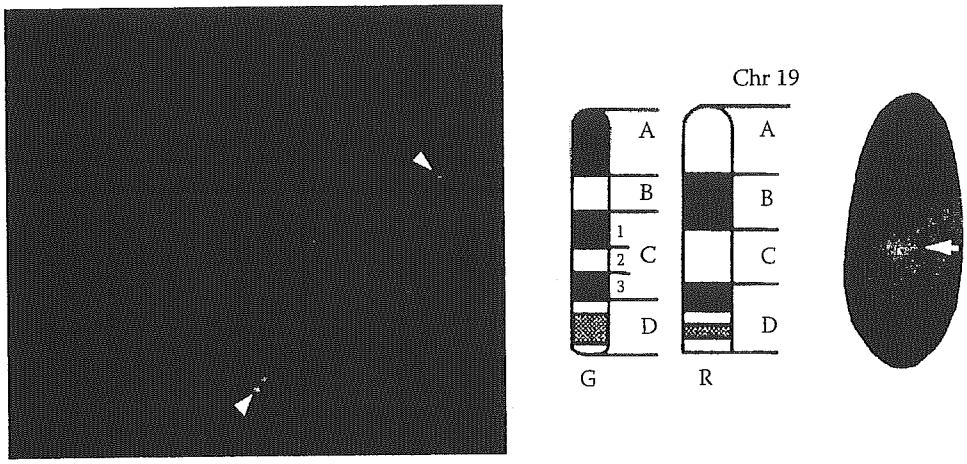


FIGURE 11.4 Chromosomal mapping of human angiotensinogen transgene performed using FISH. Chromosomes were stained with propidium iodide (PI) using the R-banding technique. On the middle of mouse Chr 19, the yellow fluorescence signals shown by a white arrowhead indicate the location of the transgene.

of a transgene by FISH. This figure demonstrates the exact chromosomal localization of the human angiotensinogen transgene on mouse Chromosome 19. We also performed gene mapping by mating experiments in order to confirm the chromosome number and to clarify the map position of transgenes. Three microsatellite DNA markers and the transgene were linked in the order: *D19Mit28*-(11.5cM)-Tg-(6.47cM)-*D19Mit40*-(0.72cM)-*D19Mit13*.[17]

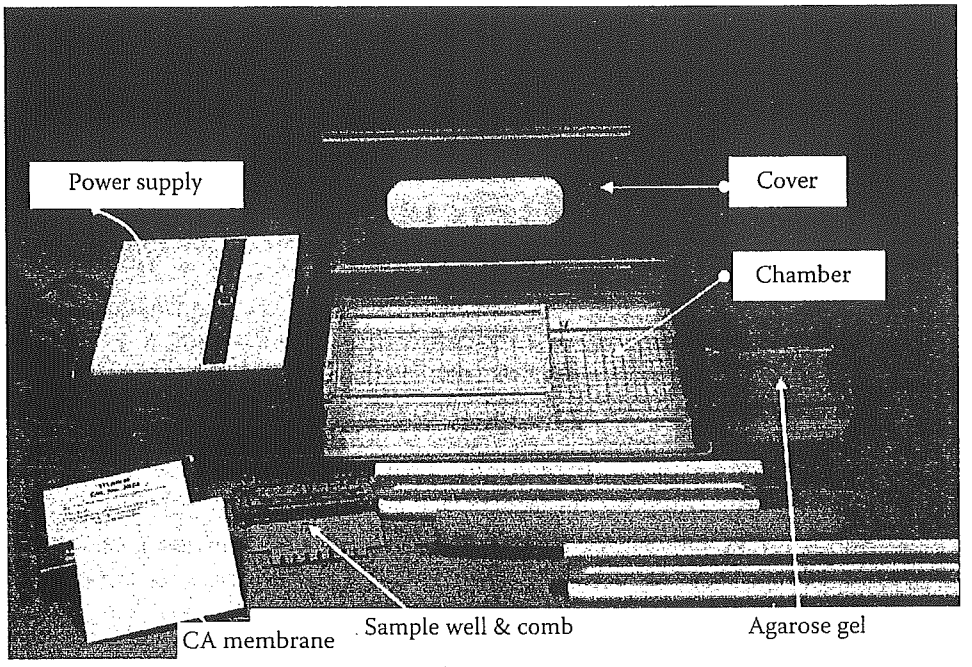


FIGURE 11.5 A genetic monitoring kit developed by the ICLAS Monitoring Center. Proteins and enzymes are detected with cellulose acetate membrane and SSLP markers with agarose gel. CA: cellulose acetate.

DEVELOPMENT OF A GENETIC MONITORING KIT

Genetic monitoring should be performed using standardized techniques. We developed a genetic monitoring kit that is useful for beginners and includes the minimum materials required and an operation manual. Protein and enzyme staining kits are supplied separately on demand. The kit can also be used as an educational tool in order to demonstrate genetic polymorphisms of proteins and enzymes in mice and rats.

CONCLUSION

Genetic monitoring was developed to ensure genetic quality of inbred strains of mice. As described in this chapter, we established a genetic monitoring system including not only genetic techniques but also procedures and concepts for testing genetic quality. Simultaneously, we successfully applied this system to test not only inbred strains of mice but also for closed colonies, cultured cell lines, sperm, and embryos.

Recently, genetic resource repositories were established worldwide. Shipping now occurs not only of live animals but also of frozen embryos and sperm preserved in liquid nitrogen. In many cases, researchers want to know the genetic background of the strain and genotype of an interesting gene immediately upon receiving such genetic resources. Based on genetic monitoring systems described

in this chapter, a procedure for assurance of genetic quality will be available at each repository or facility and utilized depending on the resources and specimens available (sperm, embryos, or live animals).

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A Mutant Mouse with Severe Anemia and Skin Abnormalities Controlled by a New Allele of the Flaky Skin (*fsn*) Locus

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Abstract: *We found a novel recessive mutation in an inbred strain, INT, that was derived from an ICR closed colony. Mice homozygous for this mutation are identified by severe anemia, dysgenesis and neonatal death. This mutation was tentatively named* int. *Intercrosses of* int *heterozygotes (+/*int*) and the flaky skin heterozygotes (+/*fsn*) resulted in abnormal mice (*int/fsn *heterozygotes) showing anemia and flaky skin with the expected frequency for autosomal recessive mutation. The* int *gene was therefore named* fsn^{Jic} *as an allele of the* fsn *locus on chromosome 17. We carried out phenotype analyses using B6.INT-* fsn^{Jic} *mice to observe phenotypes of blood and skin in the embryonic and neonatal stages. Discrimination of* fsn^{Jic} *embryos from normal embryos was performed by an indirect diagnosis of the* fsn^{Jic} *gene using the* D17Mit130 *microsatellite marker tightly linked to the* fsn *locus. The number of fetal nucleated RBC of normal embryos decreased gradually to 17.5 dpc, but that of the abnormal embryos decreased to 14.5 dpc followed by a gradual increase to 17.5 dpc. Skin of* fsn^{Jic} *embryos did not show any abnormalities and expressed cytokeratins normally as skin epithelial cell markers at each embryonic stage (15.5 dpc to 18.5 dpc). Time differences in the appearance of the different phenotypes observed in various tissue and organs of* fsn *homozygotes suggest they are caused by expression of the* fsn *gene at different developmental stages.*

Key words: *anemia, dysgenesis, flaky skin,* fsn *gene, ICR*

Introduction

It is known that many spontaneous mutations have been found in inbred strains derived from closed colonies of mice, e.g. NOD/Shi [9] and III/Jic [3, 4, 6]. In 1986, we found a novel recessive mutation identified by severe anemia and early death within 10 days of birth in the INT strain derived from a Jcl:ICR closed colony [5]. We tentatively named this mutation *int*, the same name as the strain. Genetic studies performed so far have revealed that this is an autosomal recessive mutation and that it is mapped to the distal region of

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chromosome 17.

An autosomal recessive mutation designated flaky skin (*fsn*) was found at the Jackson Laboratory (ME, USA). The *fsn* homozygous neonates show anemia and adult *fsn* homozygotes show a patchy, thick and white scale skin at about 4 weeks of age. Phenotypes of *fsn* homozygotes are associated with abnormalities of stratified squamous epithelia and the hematopoietic system. The *fsn* mutation was mapped to the distal region of chromosome 17 [1, 11, 15].

We noted similarities of the phenotypes in both *int* and *fsn* homozygotes described above. So far, we have attempted to demonstrate that these mutations are alleles of the *fsn* locus. In this paper, we describe the genetic relation between *int* and *fsn* genes demonstrated by linkage analyses and allelism tests. We also report the histological and hematological phenotypes of the affected mice homozygous for the *int* gene at the embryonic stages.

Materials and Methods

Animals and genetic crosses

A novel recessive mutation (tentatively named *int*) that occurred spontaneously was found in an inbred strain, INT, established from a Jcl:ICR closed colony at the Central Institute for Experimental Animals (Kawasaki, Japan). Mice homozygous for the *int* mutation were identified by pallor due to severe anemia at birth and skin abnormalities at about 10 days after birth.

Three *int* congenic strains, B6.INT-*int* (N8 in 2004), CBy.INT-*int* (N5 in 2004) and C3.INT-*int* (N5 in 2004), were bred using C57BL/6JJcl (B6), BALB/cByJJcl (CBy) and C3H/HeNJcl (C3) mice purchased from CLEA Japan (Tokyo, Japan). An intercross-backcross system was adapted to establish these congenic strains.

Hematological and histological studies were performed to describe the phenotypes of *int* homozygotes in the embryonic and neonatal stages. Intercrosses of B6.INT-*int* heterozygotes (+/*int*) were carried out to obtain the *int* homozygotes at 12.5, 13.5, 14.5, 15.5, 16.5, 17.5 and 18.5 dpc (days post-coitus). Observation of a vaginal plug on the morning after pairing determined the embryonic stage as 0.5 dpc. Genotypes of the *int* gene in embryos were determined using the *D17Mit130* microsatellite marker as described below.

To demonstrate allelism of *int* and *fsn* genes, crosses of B6.INT-*int* (+/*int*) and CBy.A-*fsn* (+/*fsn*) purchased from the Jackson Laboratory (ME, USA) were performed. A fine map of chromosome 17 was drawn based on the results of linkage analyses using F_2 progeny obtained by intercrosses of (B6.INT-+/*int* and C3H/HeNJcl-+/+)F_1-+/*int* mice.

Microsatellite markers for fine mapping

For fine mapping and indirect diagnosis of the *int* gene, we developed four Ham (Laboratory code for Hamamatsu University School of Medicine) microsatellite markers detected as SSLPs (simple sequence length polymorphisms) on chromosome 17 as follows: *D17Ham2* (forward: tggcccagcaagtaatgacaac, reverse tgggctggtaaaatggaccttc), *D17Ham9* (forward: actggacttcaccaacag, reverse: gctcaaagccttttcgac) and *D17Ham34* (forward: ttgtacactggcatgcct, reverse: ctggagagacaactcatcca). These primers were designed using DNA sequences obtained from the NCBI database. Other primers for microsatellite markers used in this study were purchased from Invitrogen (CA, USA).

Genomic PCR and RT-PCR

Using a standard phenol-chloroform method, genomic DNA was prepared from various tissues, depending on the experiments, and used as template DNA for PCR. Amplification of microsatellite markers by PCR was performed under the following conditions: 30 cycles of DNA degeneration for 20 s at 94°C, annealing for 30 s at 57°C and extension for 40 s at 72°C. PCR products were electrophoresed on 3% agarose gel followed by staining with ethidium bromide and the results were photographed.

RT-PCR was performed in order to observe expressions of *Hbb-bh1* (hemoglobin Z, beta-like embryonic chain) and *Hbb-b1* (hemoglobin, beta adult major chain) genes as the markers of erythrocyte maturity [8]. Total RNA was extracted from livers of 14.5 dpc embryos using RNeasy (Qiagen, MD, USA) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed using oligo-dT primer and superscript II RT (Gibco BRL, MD, USA). cDNA was amplified under the following conditions: 30 cycles of DNA degeneration for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C. Primer sequences used in this study were as follows: *Hbb-bh1* (forward: ctcaaggagacctttgctca, reverse:

agtccccatggagtcaaaga) and *Hbb-b1* (forward: cacaaccccagaaacagaca, reverse: ctgacagatgctctcttggg).

Histology and Hematology

Blood was collected in heparinized microhematocrit tubes (Drummond, PA, USA) for hematological studies. Red blood cells (RBC), white blood cells (WBC) and platelets (PLT) were counted with an automated hematology analyzer, SF-3000 (Sysmex, Hyogo, Japan). In order to observe various blood cells, blood smears were prepared and stained with Wright-Giemsa solution. The number of FNRBC (fetal nucleated red blood cells) per square millimeter was counted using NIH Image software (version 1.62 available at http://rsb.info.nih.gov/nih-image/download.html).

Tissue samples were collected and fixed in Bouin's solution overnight followed by embedding in paraffin and sectioning at 5 μm. Sections were stained with hematoxylin and eosin (HE) and observed under a light microscope (Olympus, Tokyo, Japan). Staining procedures for immunohistochemical studies were as previously described [15, 16]. Briefly, sections prepared from embryos were stained using a panel of polyclonal rabbit antibodies specific for mouse keratinocyte markers (K1, K6 and K14) [9, 10] with the ABC kit (Vector, CA, USA) according to the manufacturer's instructions. They were counterstained with hematoxylin.

Microbiological monitoring and approval of animal experimentation

Microbiological monitoring was performed by our in-house monitoring system every three months to determine microbiological conditions in the animal rooms. Samples collected from mice used in this study were sent to the ICLAS Monitoring Center (Kawasaki, Japan) to check for as many microorganisms as possible. No infection was detected in any animal room in which the mice used in this study were maintained.

The experimental protocol and design were approved by the Animal Experimentation Committee and performed according to the Guidelines for Animal Experimentation of Hamamatsu University School of Medicine.

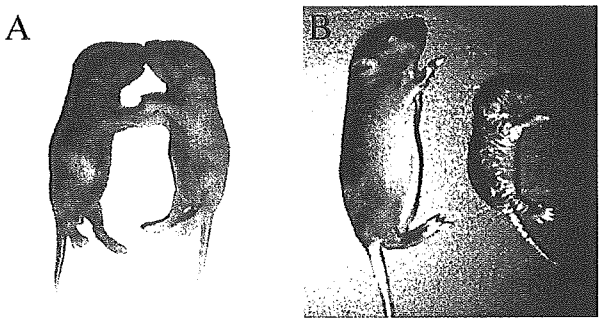

Fig. 1. Mice of congenic strains with a novel mutation showing severe anemia and skin abnormalities. (A) B6.INT-*int* (=*fsn*^{Jic}) mice at one day after birth (Right: affected mouse, Left: normal mouse). (B) C3.INT-*int* (=*fsn*^{Jic}) mice at 10 days after birth (Right: affected mouse, Left: normal mouse).

Results

Establishment of three int *congenic strains*

We bred three congenic strains of *int* gene to study phenotypes of the *int* gene on genetic backgrounds of C57BL/6JJcl, BALB/cByJJcl and C3H/HeNJcl mice.

The first congenic strain was B6.INT-*int*. The mean life span of affected mice (B6.INT-*int/int*) was 3.5 ± 0.6 days (N=15). An affected mouse one day after birth is shown in Fig. 1A (right) and a normal littermate (B6.INT-+/?: B6.INT-+/*int* or +/+) in Fig. 1A (left). Affected mice were distinguished from normal mice by abnormal phenotypes of pale skin caused by anemia and lined tails.

The second congenic strain is CBy.INT-*int* (picture not shown). The mean life span of affected mice (CBy.INT-*int/int*) was 7.7 ± 0.8 days (N=9). Affected mice showed severe anemia and papulosquamous skin lesions.

The third congenic strain is C3.INT-*int*. The mean life span of the affected mice (C3.INT-*int/int*) was 11.6 ± 0.5 days (N=7). An affected mouse at 10 days after birth is shown in Fig. 1B (right) and a normal littermate (C3.INT-+/?: +/*int* or +/+) in Fig. 1B (left). Affected mice showed severe anemia and papulosquamous skin lesions, the same as observed in the CBy.INT-*int* congenic strain.

The mean life span of the B6.INT-*int/int* mice was shorter than those of the other *int* congenic strains. A marked effect of genetic backgrounds on phenotypes of the *int* mutation was observed with the mean life span.

Inheritance of the int *gene and allelism tests*

During breeding of the three *int* congenic strains, intercrosses of *int* heterozygotes were performed to confirm inheritance of the *int* mutation. With the B6.INT-*int* congenic strain, 65 mice were obtained from seven litters. Fifteen (23.1%) mice were *int* homozygotes (9 females and 6 males). With the CBy.INT-*int* congenic strain, 25 mice were obtained from four litters, and 6 (24.0%) were *int* homozygotes (4 females and 2 males). With the C3.INT-*int* congenic strain, 123 mice were obtained from 20 litters, and 33 (26.8%) were *int* homozygotes (17 females and 16 males). Since these incidences of *int* homozygotes were identical to the incidences expected in a recessive mutation, we consider that the *int* gene reported in this paper is an autosomal recessive mutation.

To study allelism of the *int* and *fsn* genes, 37 F_1 mice were obtained by mating B6.INT-+/*int* and CBy.A-+/*fsn*. Nine (24.3%) of them showed abnormal phenotypes and 28 normal phenotypes. This incidence was identical to that (25%) expected in a recessive mutation. Therefore, it was concluded that the *int* gene is an allele of the *fsn* gene and it was tentatively named *fsn*^{Jic} according to the gene nomenclature guideline (http://www.informatics.jax.org/mgihome//nomen/gene.shtml). The mean life span of *fsn /int* (*fsn*^{Jic}) mice was 67.2 ± 3.6 days (N=9). In contrast, the mean life span of *fsn* homozygous mice (CBy.A-*fsn/fsn*) was 97.8 ± 44.8 days (N=11).

Fine map around the fsn^{Jic} *gene on chromosome 17*

Figure 2 shows the fine map around the *fsn*^{Jic} *(int)* gene on chromosome 17 drawn from the results obtained in this study. Among the Ham (Hamamatsu University School of Medicine) microsatellite markers, *D17Ham9* was mapped between *D17Mit190* and *D17Mit129*, and both *D17Ham34* and *D17Ham2* were mapped between *D17Mit130* and *D17Mit221*.

The *fsn*^{Jic} gene was precisely assigned to the distal region of chromosome 17 using 211 *fsn*^{Jic} F_2 mice obtained by intercrosses of (C3H/HeJ-+/+ × B6.INT-+/*fsn*^{Jic})F_1-+/*fsn*^{Jic}. No recombinants (0/422 chromosomes) between *fsn*^{Jic} and *D17Mit130* genes were observed. Recombination frequencies between *fsn*^{Jic} and *D17Mit129* genes and between *fsn*^{Jic} and *D17Ham34* genes were 0.2% (1/422) and 1.9% (8/422), respectively. These results suggest that the *fsn*^{Jic} gene is located between *D17Mit129* and *D17Ham34*.

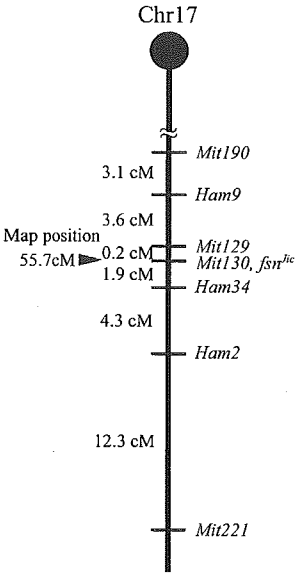

Fig. 2. Fine map of chromosome 17 drawn from the results obtained in this study. No recombinant (0/422 chromosomes) between *fsn*^{Jic} and *D17Mit130* genes was observed. Recombination frequencies between *fsn*^{Jic} and *D17Mit129* genes and between *fsn*^{Jic} and *D17Ham34* genes were 0.2% (1/422) and 1.9% (8/422), respectively. These results suggested that the *fsn*^{Jic} gene is located between *D17Mit129* and *D17Ham34*.

Characterization of the fsn^{Jic} *neonate*

Table 1 shows body weights of normal mice (+/+ or +/*fsn*^{Jic}) and *fsn*^{Jic} homozygotes (*fsn*^{Jic}/*fsn*^{Jic}) at 10 days of age and ratios of organ weights (mg) to body weights (g) in the C3.INT-*fsn*^{Jic} congenic strain. The average body weight of *fsn*^{Jic} homozygotes was lower than that of normal mice. Compared with ratios of organ weight (mg) to body weight (g) of normal mice, those of *fsn*^{Jic} homozygotes were significantly higher for the heart and spleen, but significantly lower for the thymus. Although data are not shown, we observed markedly reduced white pulp and increased red pulp in spleens of *fsn*^{Jic} homozygotes. We also observed remarkably reduced cortex cellularity in livers of *fsn*^{Jic} homozygotes. Livers and kidneys of *fsn*^{Jic} homozygotes were paler than those of normal mice. Livers of *fsn*^{Jic} homozygotes showed hepatitis with an inflammatory cell infiltrate and marked extramedullary hematopoiesis was observed

Table 1. Body and organ weights of the normal (+/+ or *fsn*^{Jic}) and affected (*fsn*^{Jic}/*fsn*^{Jic}) neonatal mice (10 days old) of the C3.INT-*fsn*^{Jic} congenic strain

Genotype	No. tested	Body weights (g)	Ratio of organ weight (mg) to body weight (g) (Organ weights (mg) in parenthesis)				
			Heart	Spleen	Thymus	Liver	Kidney
fsn^{Jic}/*fsn*^{Jic}	7	3.6 ± 0.3	10.1 ± 0.6* (38.9 ± 4.3)	8.8 ± 1.1* (32.8 ± 6.0)	1.6 ± 0.3* (5.6 ± 0.1)	48.9 ± 4.30 (177.1 ± 22)	14.6 ± 0.8 (52.2 ± 4.5)
+/?	8	7.9 ± 0.5	5.9 ± 0.8 (49.9 ± 5.4)	5.7 ± 0.6 (46.9 ± 7.2)	5.2 ± 0.3 (41.1 ± 3.5)	40.0 ± 1.17 (317.7 ± 23)	13.5 ± 1.0 (109.7 ± 8.2)

*Mean value for *fsn*^{Jic}/*fsn*^{Jic} is significantly different from +/? littermates by t-test at $P<0.05$.

Table 2. Hematologic values of the normal (+/?) and affected (*fsn*^{Jic}/*fsn*^{Jic}) neonatal mice (10 days old) of the C3.INT-*fsn*^{Jic} congenic strain

Genotype	No. tested	Hct (%)	RBC (× 10^6/ μl)	Nucleated cells (× 10^3/ μl)	MCV (fl)	MCH (pg)	MCHC (g/dl)	PLT (× 10^4/ μl)
fsn^{Jic}/*fsn*^{Jic}	5	11.3 ± 2.5**	14.7 ± 3.0**	104.0 ± 11.7**	75.3 ± 2.3	20.5 ± 1.0	27.8 ± 1.9	77.5 ± 8.3*
+/?	10	30.3 ± 0.9	42.7 ± 1.3	3.1 ± 0.3	71.0 ± 0.4	22.2 ± 0.2	31.2 ± 0.2	47.3 ± 5.4

Data are mean ± standard error of the mean. *Mean value for *fsn*^{Jic}/*fsn*^{Jic} is significantly different from +/? littermates by t-test at $P<0.01$. **Mean value for *fsn*^{Jic}/*fsn*^{Jic} is significantly different from +/? littermates by t-test at $P<0.0001$.

in livers and spleens of *fsn*^{Jic} homozygotes (data not shown).

Table 2 shows hematological data of neonates (10 days after birth) of normal and affected mice in the C3H.INT-*fsn*^{Jic} congenic strain. Hct values of *fsn*^{Jic} homozygotes were much lower than average values of normal mice, but the collected blood samples of *fsn*^{Jic} clotted normally at room temperature. The number of RBCs was significantly decreased in *fsn*^{Jic} homozygotes, but the number of nucleated cells was 35 times higher than that of normal mice. The number of platelets (PLTs) was significantly increased in *fsn*^{Jic} homozygotes. The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were almost the same in normal and affected mice.

Figures 3A and 3B show peripheral blood of a normal 10-day-old neonate and an affected 10-day-old neonate, respectively. A considerable number of the RBCs of affected neonates showed abnormalities such as dacryocytes (teardrops) and acanthocytes. Most nucleated cells observed in blood of *fsn*^{Jic} homozygous neonates were erythroblasts. It was difficult to identify types of WBC observed in blood of *fsn*^{Jic} homozygous neonates because broken (smudge) cells [2] were observed. Figures 3C and 3D show skin of a normal neonate and an *fsn*^{Jic} homozygous neonate. As shown in Fig. 3D skin disorders and sparse pelage were observed in *fsn*^{Jic} homozygous neonates with anemia. Skin of 10-day-old *fsn*^{Jic} homozygous neonates showed marked acanthosis and hyperkeratosis with focal parakeratosis and neutrophilic infiltration in epidermis associated with prominent dermal inflammatory infiltrate as shown in Figs. 3D and 3E.

Characterization of fsn^{Jic} *embryo*

In this experiment, the B6.INT-+/*fsn*^{Jic} congenic strain was used. Genotypes (+/+, +/*fsn*^{Jic} and *fsn*^{Jic}/*fsn*^{Jic}) of embryos used were indirectly determined using microsatellite markers (*D17Mit190*, *D17Mit129* and *D17Mit130*) closely linked to the *fsn*^{Jic} gene on chromosome 17.

Figure 4A shows the number of fetal nucleated red blood cells (FNRBC) in the peripheral blood of *fsn*^{Jic} homozygous embryos and normal embryos (+/+ or +/*fsn*^{Jic}) at 12.5, 13.5, 14.5, 15.5 16.5 and 17.5 dpc. The number of FNRBC in normal embryos decreased gradually to 17.5 dpc, but that in *fsn*^{Jic} homozygous embryos decreased to 14.5 dpc followed by a gradual increase to 17.5 dpc. At 17.5 dpc, the number of FNRBC in *fsn*^{Jic}

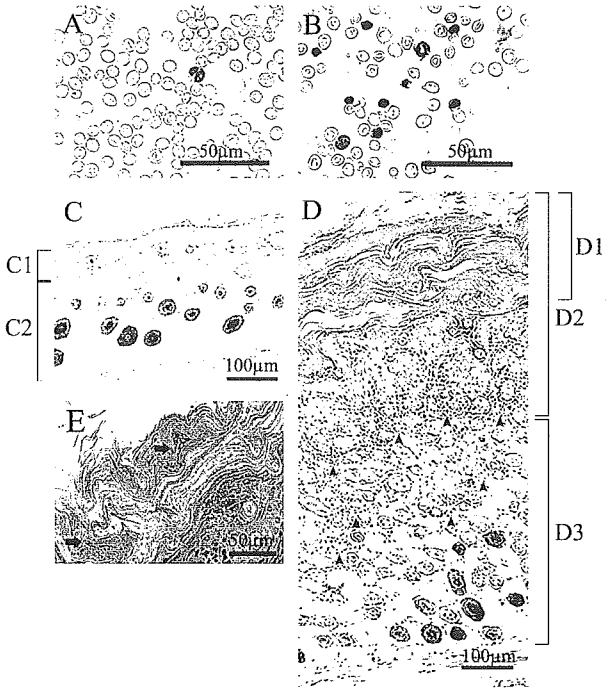

Fig. 3. Blood smears (A and B) and skins (C, D and E) of the normal (C3.INT-+/?: +/+ or +/*fsn*^{Jic}) and affected mice (C3.INT- *fsn*^{Jic}/*fsn*^{Jic}) at 10 days after birth. (A) Blood smear of the normal mouse (+/?). (B) Blood smear of the affected mouse (*fsn*^{Jic}/*fsn*^{Jic}). (C) Skin of the normal mouse (+/?); C1: Epidermis, C2: Dermis. (D) Skin of the affected mouse (*fsn*^{Jic}/*fsn*^{Jic}); D1: Corneal layer of epidermis, D2: Epidermis, D3: Dermis. Neutrophilic infiltration (shown by arrowheads) in the epidermis associated with prominent dermal inflammatory infiltrate was observed. (E) Skin of the affected mouse (*fsn*^{Jic}/*fsn*^{Jic}). Marked acanthosis and hyperkeratosis with focal parakeratosis was observed as shown by arrows.

embryo was 20 times higher than that of normal embryos. Figure 4B shows blood cells of normal and *fsn*^{Jic} homozygous embryos at 12.5, 13.5, 15.5 and 16.5 dpc. We demonstrated that blood in normal and *fsn*^{Jic} homozygous embryos did not show any histological differences at 12.5 dpc, but the number of FNRBC increased with age (Fig. 4A).

Figure 5 shows dorsal skin sections of normal and *fsn*^{Jic} homozygous embryos at 15.5, 16.5 and 18.5 dpc. Skin of *fsn*^{Jic} homozygous embryos showed no gross histological abnormalities. Figure 6 shows distributions of three keratinocyte markers, K6, K1 and K14, in skin of normal and *fsn*^{Jic} homozygous mice. Periderm was stained with anti-K6 antibody (Figs. 6A and 6B), the upper half of the non-cornified epidermis and stratum spinosum was stained with anti-K1 (Figs. 6C and 6D) and the lower half of the epidermis and stratum basale was stained with anti-K14 (Figs. 6E and 6F). There was no difference in distribution of the three keratinocyte markers and thickness of the three keratinocyte layers stained between normal and *fsn*^{Jic} homozygous embryos.

Discussion

Mapping data derived in this study show that the *int* (*fsn*^{Jic}) mutation is located between *D17Mit129* and *D17Ham34* and that no recombinant occurred between *fsn*^{Jic} and *D17Mit130*. Therefore, we assumed that the *D17Mit130* marker is useful for indirect diagnosis of genotypes of the *fsn*^{Jic} gene. In this study, we successfully performed various phenotypic analyses using *fsn*^{Jic} homozygous embryos diagnosed for this marker.

Sundberg *et al.* [15] reported that A/J-*fsn* and B6.A-*fsn* exhibit severe phenotypes and die before weaning, while CBy.A-*fsn* mice are mildly anemic at birth and survive until about 3 months of age. In order to obtain *fsn*^{Jic} mice with mild phenotypes and a longer mean life span, we established three congenic strains, B6.INT-*fsn*^{Jic}, CBy.INT-*fsn*^{Jic} and C3.INT-*fsn*^{Jic}. As a result, we observed that phenotypes of B6.INT-*fsn*^{Jic} mice are more severe (mean life span: 3.5 ± 0.6 days) than those of C3.INT-*fsn*^{Jic} mice (mean life span: 11.6 ± 0.5 days). These results reveal that genetic backgrounds have critical effects on phenotypes of the *fsn*^{Jic} gene. The CBy.INT- *fsn*^{Jic} congenic strain with the same genetic background (BALB/cByJ) as the CBy.A-*fsn* congenic strain with a longer mean life span (97.8 ± 44.8 days) showed a short mean life span (7.7 ± 0.8 days). This strongly suggests that the *fsn* and *fsn*^{Jic} genes might have different types of mutations, e.g., insertions or deletions.

Histological studies of *fsn*^{Jic} homozygotes showed severe papulosquamous skin lesions and hematopoietic system disorders as shown in Fig. 1. The *fsn*^{Jic} homozygotes showed enlarged hearts and spleens and reduced weights of thymuses. Beamer *et al.* [2] also observed the same phenotypes in *fsn* homozygotes. The reduced weights in thymuses and the reduced dimensions of the white pulp of spleens in *fsn*^{Jic} homozygotes indicate a decrease in the number of lymphocytes. On the other hand, enlargement of red pulp in spleens of

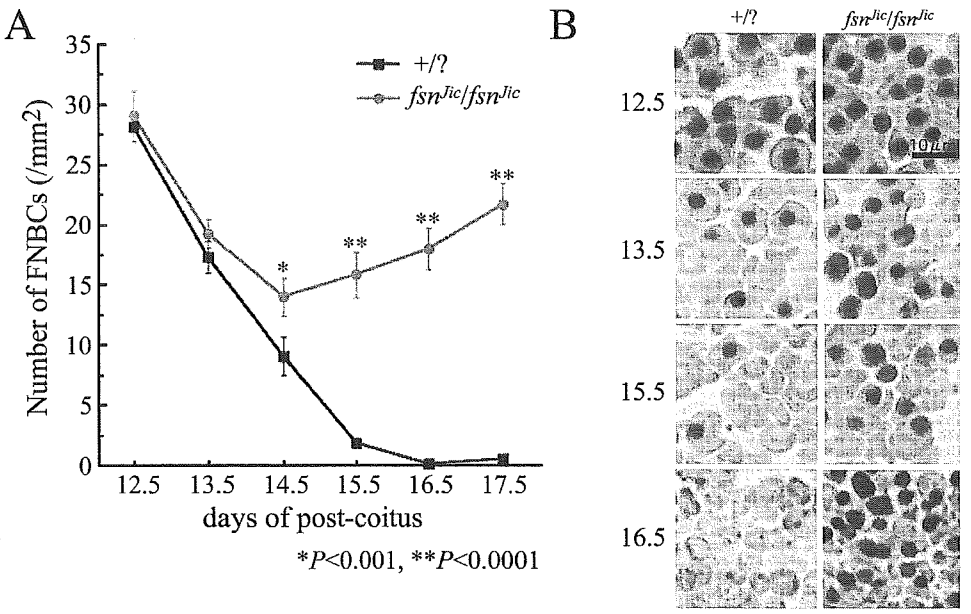

Fig. 4. Phenotypes of fetal nucleated blood cells in the normal and *fsn*^{Jic} homozygous mice at 12.5 dpc to 17.5 dpc. (A) Fetal nucleated red blood cells (FNRBC) in the peripheral blood of the normal and affected mouse. The number of FNRBC in the affected mice was decreased from 12.5 dpc to 14.5 dpc, but increased gradually to 17.5 dpc. (B) Blood of normal and affected mice. The numbers of nucleated primitive erythrocytes in the peripheral blood of normal and *fsn*^{Jic} homozygous embryos at 12.5 dpc were almost the same.

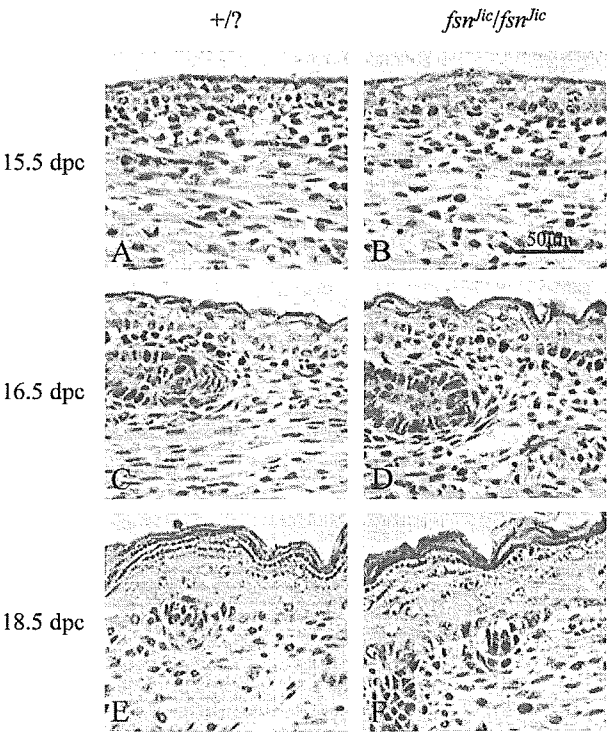

Fig. 5. Skin of *fsn*^{Jic} homozygous embryos at 15.5, 16.5 and 18.5 dpc.

fsn^{Jic} homozygotes indicates an increase in the number of erythroblasts. The cause of the enlarged hearts in *fsn*^{Jic} homozygotes is unknown. As shown in Table 2, peripheral blood collected from *fsn*^{Jic} homozygotes showed increases of nucleated cells (including erythroblasts and WBC) and PLT and reductions in Hct and RBC. It has been reported that *fsn* homozygotes show increases of B cells, macrophages, mast cells, eosinophils and immature erythroid cells (erythroblasts and reticulocytes) and decreases of T cells, lymphocytes, monocytes, neutrophils and mature RBC [2, 12]. These results suggest that *fsn* homozygotes have abnormal differentiation of hematopoietic stem cells.

In order to study hemopoiesis of *fsn*^{Jic} homozygotes, we focused on the phenotypes of B6.INT-*fsn*^{Jic} mice at various embryonic stages. As shown in Fig. 5, skin of *fsn*^{Jic} homozygotes stained with HE did not show any disorders at any embryonic stage. Normal distribution of cytokeratins, epithelial cell markers in the skin of *fsn*^{Jic} homozygous embryos, was also observed (Fig. 6). In contrast, peripheral blood of *fsn*^{Jic} homozygotes in the early embryonic stages already showed abnormali-