Induction of Skin Papillomas, Carcinomas, and Sarcomas in Mice in Which the Connexin 43 Gene is Heterologously Deleted

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It has been suggested that blocked gap junctional intercellular communication plays a crucial part in multistage carcinogenesis. The mouse skin tumor-promoting phorbol esters are potent inhibitors of gap junctional intercellular communication and this inhibition is considered to be a mechanism by which clonal expansion of ''initiated'' cells is promoted. We examined whether mice in which the gene for a gap junction protein, connexin 43, is heterozygously deleted are more susceptible to chemical carcinogenesis; connexin 43 is expressed in the basal cell layer and the dermis of the skin. When the back skin was painted with 7,12-dimethylbenz[*a*]anthracene and 12-*O*-tetradecanoylphorbol 13-acetate, the incidence and yields of both papillomas and carcinomas were similar in connexin $43^{+/-}$ and connexin $43^{+/+}$ mice; for this experiment, the original mice with C57BL/6 genetic background was crossed with CD1 strain for three generations. Subcutaneous injection of 7,12-dimethylbenz[*a*]anthracene resulted in induction of fibrosarcomas in connexin $43^{+/-}$ and connexin $43^{+/+}$ mice to a similar extent. All papillomas and carcinomas induced with 7,12-dimethylbenz[*a*]anthracene and 12-O-tetradecanoylphorbol 13-acetate contained the 7,12-dimethylbenz[*a*] anthracene-specific mutation in the *ras* gene (A to T transversion at the 61st codon). About 50% of fibrosarcomas also contained this mutation, but in the Ki-*ras* gene; there was no difference in the prevalence of this mutation in tumors from connexin $43^{+/-}$ and connexin $43^{+/+}$ mice. None of the tumors examined, however, showed any mutation in the connexin 43 gene. These results suggest that the deletion of one allele of the connexin 43 gene does not significantly contribute to, nor alter, the molecular events involved in skin carcinogenesis. These results are compatible with previous observations that nongenetic disruption of function rather than mutations of connexins, commonly occurs in cancer cells. ***Key words: gap junctional intercellular communication/two-stage carcinogenesis.***
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Gap junctional intercellular communication (GJIC) is believed to play a key part in the maintenance of homeostasis by keeping the level of second messengers equal among gap junction-connected cells in a given tissue. The gap junction channels are considered to be ''connexons'' composed of hexamers of proteins called connexins and so far, 13 different connexin genes have been identified (Bruzzone *et al*, 1996; Goodenough *et al*, 1996). Reflecting the importance of GJIC in homeostasis, mutations of connexin genes are associated with various human diseases, such as X-linked Charcot–Marie–Tooth syndrome (Bergoffen *et al*, 1993), hereditary nonsyndromic deafness (Kelsell *et al*, 1997; Xia *et al*, 1998), and heterotaxia (Britz-Cunningham *et al*, 1995). Mutation of the connexin (Cx) 31 gene has also been reported to predispose patients to a skin disease, erythrokeratodermia variabilis (Richard *et al*, 1998). In addition, the essential roles of Cx genes in differentiation and development have been proven in Cx43-deficient (knock-out) and Cx43-transgenic mice (Reaume *et al*, 1995; Ewart *et al*, 1997), Cx46-deficient mice (Gong *et al*, 1997), Cx37-deficient mice (Simon *et al*, 1997), Cx40-deficient mice (Kirchhoff *et al*, 1998), and Cx26-deficient mice (Gabriel *et al*, 1998), all of which show developmental embryonic death or malfunction of specific tissues.

Based on the idea that deviation from normal homeostasis is a key event in carcinogenesis, the role of aberrant GJIC has long been postulated (Yamasaki and Naus, 1996; Trosko and Ruch, 1998). Since the discovery that a human tumor lacked GJIC capacity (Loewenstein and Kanno, 1966), this has been confirmed and extended in numerous studies, so that aberrant GJIC is now regarded as a hallmark of cancer cells (Yamasaki and Naus, 1996). GJIC in cultured cells as well as in rat liver and mouse skin *in vivo* is inhibited by promoting agents (Yamasaki, 1996). Disappearance of gap junctional structures in mouse skin *in vivo* after treatment with the tumor-promoting agent 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has also been reported (Kalimi and Sirsat, 1984). Cells transformed by chemical carcinogens or oncogenes such as v-*ras*, v-src, and v-raf display loss of homologous or heterologous GJIC (Enomoto and Yamasaki, 1984; Azarnia *et al*, 1988; Bignami *et al*, 1988). When a connexin gene is transfected into transformed or

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Abbreviations: Cx, connexin; DMBA, 7,12-dimethylbenz[*a*] anthracene; GJIC, gap junctional intercellular communication.

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cancerous cells, tumor formation, cell growth, or both are suppressed (Kalimi *et al*, 1992; Eghbali *et al*, 1991; Rose *et al*, 1993; Mesnil *et al*, 1995). Inhibition of heterologous GJIC by transfection of anti-sense Cx43 into normal cells enhances tumor formation (Goldberg *et al*, 1994), and restoration of heterologous GJIC in transformed cells by chemicals (Yamasaki and Katoh, 1988) or by transfection of a connexin gene suppresses cell transformation (Esinduy *et al*, 1995). In some experimental tumors, mutations of connexin genes have been reported (Omori *et al*, 1996; Saito *et al*, 1997). These results suggest that intact homologous and/or heterologous GJIC is necessary for normal cell growth control and that connexin genes have tumor-suppressive effects.

Recent experiments using Cx32 gene knock-out mice have provided more direct evidence for the involvement of aberrant GJIC in carcinogenesis. Mice lacking the Cx32 gene, one of the major connexins in liver, had 8–25-fold more spontaneous liver tumors per mouse than wild-type mice (Temme *et al*, 1997). In addition, when such mice were treated with the carcinogen diethylnitrosamine, liver tumors appeared at earlier times and were larger than the spontaneous tumors. In order to determine whether an effect of a specific connexin gene deletion could also clearly be seen in skin carcinogenesis, we employed Cx43-deficient mice, as the Cx43 gene is expressed in the basal layer of the skin as well as in the dermis (Butterweck *et al*, 1994). Abnormal expression of Cx43 in skin tumors has already been reported (Budunova *et al*, 1995; Kamibayashi *et al*, 1995; Sawey *et al*, 1996). As $Cx43^{-/-}$ mice are not viable, we used $Cx43^{+/-}$ mice; such heterozygous mice can be considered as germ-line carriers of loss of heterozygosity in terms of the Cx43 locus which may be more vulnerable to carcinogenic insults. We induced epidermal tumors as well as dermal tumors in these mice with 7,12-dimethylbenz[*a*] anthracene (DMBA) and compared the results with those from wild-type control mice.

MATERIALS AND METHODS

Source of Cx43-null mice and genotyping of Cx43 gene by polymerase chain reaction (PCR) Cx43-deficient mice were originally established by Reaume *et al* (1995) and we purchased them from Jackson Laboratories (Paris).

As Cx43-nullzygous mice die just after birth due to cardiac malformation (Reaume *et al*, 1995), heterozygous mice were used for breeding. The original genetic background of these mice was C57BL/6, and we crossed them with a CD1 mice to generate heterozygous Cx43-deficient mice with CD1 genetic background, as C57BL/6 are known to be resistant to epidermal carcinogenesis. For identification of the genotype of the mice, a small piece of tail was digested in 0.7 ml of extraction buffer (0.1 M NaCl, 0.05 M Tris–HCl, 0.1 M ethylenediamine tetraacetic acid, 0.49 mg protease K per ml, 1% sodium dodecyl sulfate) at 37°C overnight. After shaking, 0.25 ml of 5 M NaCl was added and mixed well, then samples were centrifuged. About 0.7 ml of the aqueous phase was transferred into a new tube and 0.5 ml of isopropanol was added to precipitate DNA. After centrifuging, DNA was washed with 70% ethanol and dissolved in 0.5 ml of TE buffer. DNA from each mouse was analyzed by PCR with primers for neo genes as described previously (Yamakage *et al*, 1998)

Treatment of mice with carcinogens For the two-stage carcinogenicity study by skin painting, female $Cx43^{+/+}$ and $Cx43^{+/-}$ mice were used, which had been crossed with CD1 mice for three generations. We also included female C57BL/6 and CD1 mice as reference controls. The dorsal lumbar region of 7 wk old mice was clipped clean of fur with an electric hair clipper. After clipping, each mouse was painted with 10 μg DMBA (Sigma, St Louis, MO) or acetone (0.1 ml) twice a week for 5 wk followed by painting with 10 μg TPA (Chemsyn Science Lab., Lenexa, Kansas) or acetone (0.1 ml) twice a week for more than 40 wk. Fifteen mice were used in each group; the mice were observed daily and tumor appearance and numbers were recorded weekly.

In order to induce fibrosarcomas, 12–15 wk old male mice with C57BL/6 genetic background were subcutaneously injected once with 250 μg DMBA at two dorsal regions.

PCR–single strand conformation polymorphism analysis of Cx43 gene The Cx43 gene was screened for mutations by PCR–single strand conformation polymorphism analysis (Omori *et al*, 1996; Saito *et al*, 1997). A reaction mixture composed of sample DNA, PCR buffer, Taq polymerase, primers, dNDP, and [α-33]dCTP was prepared, then PCR amplification of the Cx43 gene was performed in sequential cycles (35 cycles) at 94°C for 1 min, 61°C for 1 min 50 s and 72°C for 1 min 30 s, followed by incubation for 10 min at 72°C. Six pairs of primers were used to cover all exons of the Cx43 gene as shown in **Table I**.

Table I. Pairs of primers used to cover all exons of the Cx43 gene

Primer 1	sense	5′-TGTTTTTCTTAGGAGGTGCC-3′
Primer 1	anti-sense	5′-GTTACAGCGAAAGGCAGACT-3′
Primer 2	sense	5′-TTTTCAGAATCCTGCTCCTG-3′
Primer 2	anti-sense	5′-GCTTCTCTTCCTTTCTCATC-3′
Primer 3	sense	5′-ACACTCCTGTACTTGGCTCAC-3′
Primer 3	anti-sense	5′-CGAAGACAGACTTGAAGAGG-3′
Primer 4	sense	5′-TGAGAACCTACATCATCAGC-3′
Primer 4	anti-sense	5′-GATCCTTAACGCCCTTGAAG-3′
Primer 5	sense	5′-GGTGTCTCTCGCTCTGAATA-3′
Primer 5	anti-sense	5′-TTTTGCTCGCTGGCTTGCTT-3′
Primer 6	sense	5′-ATTCCTCCTGCCGCAATTAC-3′
Primer 6	anti-sense	5′-CAGCTTGATGTTCAAGCCTG-3′

PCR products were mixed with same volume of F-dye, then denatured for more than 5 min at 94°C, 4 μl of samples were applied to 6% polyacrylamide gel (acrylamide: bisacrylamide, 99:1) with 5% glycerol. After electrophoresis, the gels were dried and exposed to X-ray film (Biomax; Eastma Kodak).

Detection of A to T mutation in Ki-*ras* and H-*ras* gene at the 61st codon The A to T mutation at the 61st codon of the Ki-*ras* and Ha-*ras* gene was analysed by PCR–restriction fragment length polymorphism as described previously (Sasaki *et al*, 1995). The primers used for the Ki-*ras* gene were 5′-GACTCCTACAGGAAACAAGT and 5′-CTATAATGGTGAATATCTTC and those for the H-*ras* gene were 5′-GACTCCTACCGGAAACAGGT and 5′-AGGAAGCCCTCCCCTGTGCG. The PCR products were digested with *Xba*I; these digested products were then loaded on to 6% acrylamide gel. An A to T mutation at this codon in both *ras* genes creates an *Xba*I digestion site (Bizub *et al*, 1986).

RESULTS

Two-stage skin carcinogenesis by painting of DMBA and TPA The skin tumor incidence and yields in various mouse lines after painting with DMBA and/or TPA are summarized in **Table II**. As expected, C57BL/6 mice were more resistant to skin carcinogenesis induced by DMBA and TPA. We crossed the $Cx43^{+/-}$ mice of C57BL/6 background with CD1 mice three times to create more susceptible transgenic mice. Theoretically, this will not completely convert the genetic background. As the incidence and yield of papillomas in $Cx43^{+/+}$ mice were similar to those found in wild-type CD1 mice, however, we conclude that the genetic background of our $Cx43^{+/+}$ mice was almost completely converted from that of C57BL/6 to CD1 in terms of their susceptibility to epidermal carcinogenesis. There was no significant difference in sensitivity between $Cx43^{+/+}$ and $Cx43^{+/-}$ mice for skin papilloma induction by DMBA/TPA, but there were more papillomas in $Cx43^{+/+}$ than $Cx43^{+/-}$ mice after painting with TPA alone. We did not observe any tumors in any mouse line treated with DMBA alone. Some skin papillomas were converted into carcinomas and the rate of such conversion was again similar in $Cx43^{+/+}$ and $Cx43^{+/-}$ mice (**Table II**).

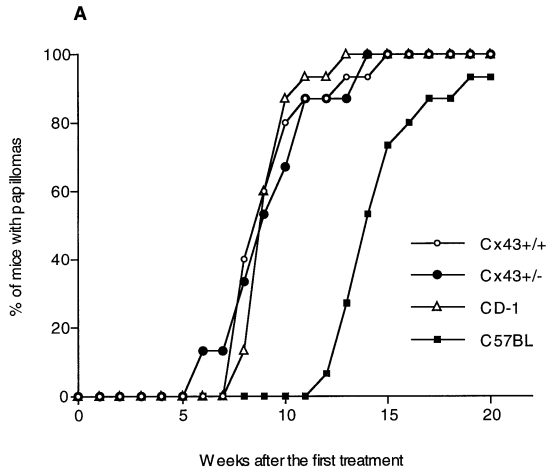
The time course of skin papilloma induction by DMBA and TPA painting is shown in **Fig 1**. It is again clear that there was no difference between $Cx43^{+/+}$ and $Cx43^{+/-}$ mice in the papilloma induction rate.

Induction of tumors after subcutaneous injection of DMBA The first fibrosarcoma appeared in $Cx43^{+/-}$ mice 15 wk after subcutaneous injection of DMBA. The appearance of the first fibrosarcoma was 1 wk later in $Cx43^{+/+}$ mice, but the percentage of mice bearing a tumor reached the same level 2 wk later (**Fig 2**). As shown in **Table III**, the final percentage of mice bearing tumors was very similar between $Cx43^{+/+}$ and $Cx43^{+/-}$ mice. Although $Cx43^{+/-}$ mice showed only fibrosarcomas, the

Table II. Incidence and yield of skin tumors induced by painting with DMBA and/or TPA on Cx43$^{+/+}$ and Cx43$^{+/-}$ mice [a]

Genotype of Cx43	Initiation	Promotion	No. of mice used	No. ot tumors		No. of mice with	
				Papilloma	Carcinoma	Papilloma	Carcinoma
+/+[b]	Acetone	Acetone	15	0	0	0	0
	Acetone	TPA	15	26	1	10	1
	DMBA	Acetone	15	0	0	0	0
	DMBA	TPA	15	401	1	15	1
+/–[b]	Acetone	Acetone	15	0	0	0	0
	Acetone	TPA	15	11	0	6	0
	DMBA	Acetone	15	0	0	0	0
	DMBA	TPA	15	333	3	15	3
+/+ (original CD-1)	Acetone	Acetone	15	0	0	0	0
	DMBA	TPA	15	345	2	15	2
+/+ (originalC57BL/6)	Acetone	Acetone	15	0	0	0	0
	DMBA	TPA	15	55	1	14	1

[a]Experimental details are described in *Materials and Methods*.
[b]Original background strain was C57BL. For this experiment, they were crossed with CD-1 mice. The experiments were performed with offsprings after crossing with CD-1 three times.



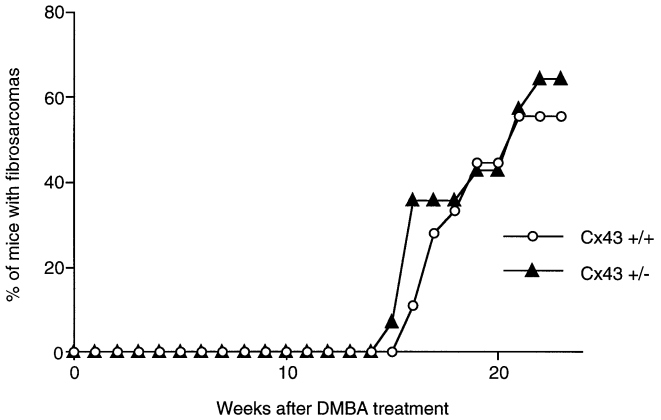


Figure 2. Time course of fibrosarcoma production in Cx43$^{+/+}$ and Cx43$^{+/-}$ mice after subcutaneous injection of DMBA. Experimental details are described in *Materials and Methods*.

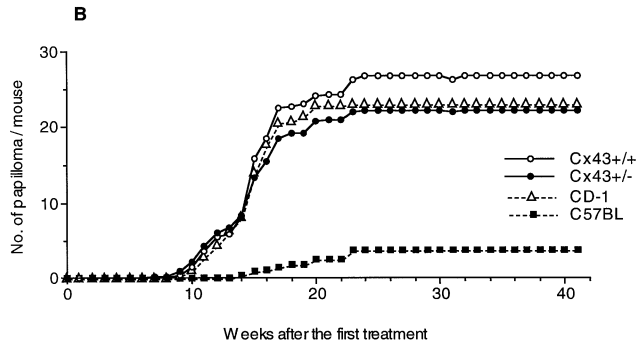


Figure 1. Time course of skin papilloma appearance in Cx43$^{+/+}$ and Cx43$^{+/-}$ mice after painting with DMBA and TPA. Experimental details are described in *Materials and Methods*. (*A*) Incidence curve. (*B*) Yield curve. Cx43$^{+/+}$ and Cx43$^{+/-}$ mice were derived after original Cx43$^{+/-}$ with C57BL background were crossed with CD1 three times.

Cx43$^{+/+}$ mice had two rhabdomyosarcomas, a liposarcoma and an anaplastic sarcoma (**Table III**).

Mutation of Ha-*ras*, Ki-*ras*, and Cx43 genes in tumors We examined the presence of Cx43 gene mutations in tumors induced by DMBA and/or TPA in various mouse lines. We specifically considered the possibility that the remaining allele of the Cx43 gene in Cx43$^{+/-}$ mice might be a target for DMBA mutagenesis. Examination of mutations by single strand conformation polymorphism analysis of the whole coding region of the Cx43 gene revealed no mutation in papillomas, carcinomas or fibrosarcomas that developed in any line of mice (**Table IV**).

When skin tumors are induced by DMBA, many of them contain a specific A to T transversion at the 61st codon of the Ha-*ras* or Ki-*ras* gene. In papillomas and carcinomas, the Ha-*ras* gene is usually mutated, whereas the Ki-*ras* gene is mutated in fibrosarcomas (Bizub *et al*, 1986; Sasaki *et al*, 1995). We were interested in whether the absence of one allele of Cx43 gene in Cx43$^{+/-}$ mice would affect the molecular events of DMBA-induced carcinogenesis. PCR/restriction fragment length polymorphism analysis revealed the presence of A to T mutations at the 61st codon of the Ha-*ras* gene in papillomas and carcinomas. On the other hand, only Ki-*ras* gene mutations were observed in fibrosarcomas, confirming our earlier results (**Fig 3**). As summarized in **Table IV**, the prevalence of Ha-*ras* and Ki-*ras* gene mutations in papillomas or carcinomas and fibrosarcomas, respectively, was

Table III. Incidence of fibrosarcomas in mice subcutaneously injected with 250 μg of DMBA[a]

Genotype of Cx43	Initiation	No. of mice used	No. of injections	No. of fibrosarcomas (%)	No. of other malignant tumors (%)
+/+	Olive oil	10	20	0 (0)	0 (0)
	DMBA	9	18	10 (55.6)	4[b] (22.2)
+/–	Olive oil	9	18	0 (0)	0 (0)
	DMBA	7	14	9 (64.3)	0 (0)

[a]Experimental details are described in *Materials and Methods*.
[b]Two rhabdomyosarcomas, one liposarcoma, one anaplastic sarcoma.

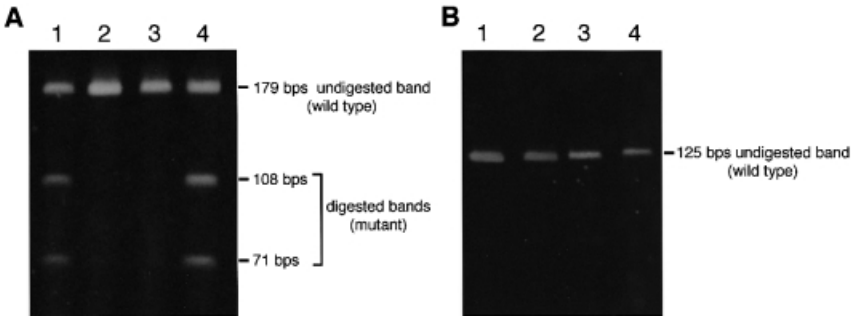


Figure 3. Analysis of Ki-*ras* and Ha-*ras* mutations (A to T transversion at the 61st codon) by PCR/restriction fragment length polymorphism method. (*A*) Ki-*ras* mutations; (*B*) Ha-*ras* mutations. Tumor DNA samples were subjected to the PCR/restriction fragment length polymorphism analysis as described in *Materials and Methods*. (1, 2) Fibrosarcomas that developed in $Cx43^{+/+}$ mice; (3, 4) fibrosarcomas that developed in $Cx43^{+/-}$ mice.

Table IV. Cx43, Ki-, and Ha-*ras* mutations in fibrosarcomas, papillomas and carcinomas[a]

Tumor	Genotype of Cx43	No. of mutations/No. of tumors analysed		
		Cx43	Ki-*ras* (%)	H-*ras* (%)
Fibrosarcoma[b]	+/+	0/10 (0.0)	8/10 (80.0)	0/10 (0.0)
	+/–	0/9 (0.0)	5/9 (55.6)	0/9 (0.0)
	Total	0/19 (0.0)	13/19 (68.4)	0/19 (0.0)
Papilloma[c]	+/+	0/16 (0.0)	NT	6/6 (100.0)
	+/–	0/13 (0.0)	NT	9/9 (100.0)
	Total	0/29 (0.0)		15/15 (100.0)
Carcinoma[c]	+/+	0/2 (0.0)	NT	1/1 (100.0)
	+/–	0/1 (0.0)	NT	NT
	Total	0/3 (0.0)		1/1 (100.0)

[a]Mutation analysis was performed as described in *Materials and Methods*.
[b]Tumors from the experiment presented in **Table III**.
[c]Tumors from the experiment presented in **Table II**.

similar among those that developed in mice with different Cx43 genotypes. These results suggest that the absence of one allele of the Cx43 gene did not affect the molecular mechanisms involved in DMBA-induced carcinogenesis.

DISCUSSION

We have shown here that $Cx43^{+/-}$ mice are not more susceptible than $Cx43^{+/+}$ mice to skin carcinogenesis after DMBA/TPA painting and to fibrosarcoma production by subcutaneous injection of DMBA. As none of the tumors induced in $Cx43^{+/-}$ mice carried mutations in the remaining allele of the Cx43 gene, it is likely that loss of Cx43 gene function as a result of mutations is not a major pathway of DMBA-induced skin carcinogenesis. This is in line with our previous finding that connexin gene mutations occur rarely in tumors: we detected only one Cx32 mutation in a rat liver tumor and one Cx37 mutation in a rat hemangiosarcoma (Omori *et al*, 1996; Saito *et al*, 1997). In fact, we have found no mutations in the Cx32, Cx37, and Cx43 genes in human liver, stomach, lung, and breast cancers, nor in meningiomas (Krutovskikh *et al*, 1994, 1996; Mironov *et al*, 1994; Sato *et al*, 1997). On the other hand, aberrant expression and/or localization of connexins are often seen in cancerous cells (Trosko and Ruch, 1998). Indeed, aberrant expression of Cx43 has been observed during mouse skin carcinogenesis (Budunova *et al*, 1995; Kamibayashi *et al*, 1995; Sawey *et al*, 1996). Therefore, it is likely that the function of connexins is not generally impaired through mutations, but rather through the level of their expression and as a result of post-translational modifications during carcinogenesis.

As $Cx43^{-/-}$ mice do not survive after birth, it was not possible to include them in our experiments. We previously established fibroblast cell lines, however, by the 3T3 method from embryos of such mice and examined their growth properties (Yamakage *et al*, 1998). Our results suggest that there was no significant difference in the immortalization process and in the growth characteristics of 3T3 fibroblasts established from $Cx43^{+/+}$ and $Cx43^{-/-}$ mice (Yamakage *et al*, 1998). We thus concluded that Cx43 gene itself has weak growth control ability by itself. On the other hand, it has been clearly shown that the Cx43 gene can control growth of already transformed or tumorigenic cells (Rose *et al*, 1993; Krutovskikh *et al*, 1998). It has also been shown that $Cx43^{-/-}$ mouse embryo fibroblasts established under conditions other than the 3T3 method acquired some transformed phenotypes (Martyn *et al*, 1997). These results suggest that Cx43, and possibly also other connexins, may exert growth control ability when cells are under certain constraints. This is compatible with the idea that GJIC is essential for the maintenance of homeostasis and we propose that GJIC is involved in growth regulation through such a process.

Analysis of *ras* gene mutations in tumors revealed the typical molecular signature of DMBA, i.e., A to T transversion at the 61st codon. We also confirmed that only Ha-*ras* gene mutations were seen in papillomas and carcinomas, whereas fibrosarcomas contained only Ki-*ras* gene mutations. These results are consistent with our previous suggestion that DMBA may induce the same mutation in both Ha-*ras* and Ki-*ras* genes in both epidermal and dermal cells, but that their recruitment processes are different in these two cell types (Sasaki *et al*, 1995). As both $Cx43^{+/+}$ and $Cx43^{+/-}$ mice bore tumors with the same pattern of *ras* gene

mutations, loss of one Cx43 gene allele does not seem to influence this recruitment process.

This conclusion is also reinforced by the finding that Cx43$^{+/-}$ mice developed fewer papillomas than Cx43$^{+/+}$ mice when painted by TPA alone; in this experimental group, TPA is likely to promote the growth of ''spontaneously'' initiated cells. On the other hand, it is difficult to explain why Cx43$^{+/-}$ mice developed fewer papillomas.

Most cell types express more than one type of connexin gene. In rodent and human keratinocytes, besides Cx43, Cx26, Cx30.3, Cx31, Cx31.1, Cx37, Cx40, and Cx45 genes are expressed (Salomon *et al*, 1993; Willecke *et al*, 1993; Butterweck *et al*, 1994; Goliger and Paul, 1994). As it has been shown that different connexins form gap junctions which have different conductance and as some different connexins are known to form heteromeric connexons (Stauffer, 1995; Jiang and Goodenough, 1996), it is possible that the presence of so many different connexin genes is designed to allow the fine-tuning of intercellular communication. On the other hand, it is also possible that multiple connexin genes are there to provide compensatory activity when any connexin is damaged. If this is the case, it is not surprising that Cx43$^{+/-}$ mice are not more prone to carcinogenesis, as other connexins may be able to maintain fairly normal function of keratinocytes and dermal cells.

So far, an increased incidence of tumors in mice has been associated only with a deficiency of the Cx32 gene (Temme *et al*, 1997). In this mouse line, the higher incidence of tumors was limited to the liver (Temme *et al*, 1997). In the hepatocytes, the Cx26 and Cx32 genes are expressed. In the Cx32-nullzygous mice, there was also a drastic decrease in Cx26 gene expression in the hepatocytes and an increased rate of DNA synthesis in the liver, as measured by bromodeoxyuridine labelling compared with wild-type mice (Temme *et al*, 1997). This may support the concept outlined above that a deficiency of one connexin is not sufficient to contribute strongly to carcinogenesis, but that a more general reduction in GJIC may affect growth control.

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