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Altered tumor biology and tumorigenesis in irradiated and chemical carcinogen-treated single and combined Connexin32/p27^{Kip1}-deficient mice

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Abstract

Connexin32 knockout mice (Cx32-KO) exhibit increased chemical and radiation-induced liver and lung tumorigenesis. This increased tumor incidence is associated with altered tumor biology including enhanced tumor progression and an increased percent of MAPK-active tumors. Likewise, mice lacking the tumor suppressor/cell cycle regulator p27^{Kip1} exhibit increased tumorigenesis in a variety of tissues following chemical and radiation induction. Interestingly, in a double-deficient mouse model (DKO), additional loss of p27^{Kip1} in a Cx32-KO background results in attenuation of liver and lung tumorigenesis as well as MAPK activation profiles suggesting pathway interaction. While these mouse strains exhibit altered liver and lung tumor susceptibility following both chemical (DEN) and radiation (X-ray) induction protocols, comparisons of the resulting tumor incidence, multiplicity, tumor progression and MAPK activation in response to these two distinct carcinogens underscores the separate influence of each individual gene on both tumor formation and activation of specific oncogenic pathways. Furthermore, these studies demonstrate that different carcinogens interact disparately with Cx32/p27^{Kip1} genotypic backgrounds *in situ* resulting in varied tumorigenic response.

Keywords

gap junction; connexin; p27^{Kip1}; radiation; DEN; lung; liver; carcinogenesis; Connexin32; mouse; knockout (KO)

INTRODUCTION

Vertebrate gap junction proteins, connexins, are a family of highly conserved proteins with 20 members identified in rodents and 21 in humans that demonstrate developmental as well as tissue-specific expression patterns (Stout et al., 2004; Willecke et al., 2002). Gap junction intercellular communication (GJIC) allows for the direct transmission of ions and small hydrophilic molecules less than 1–2 kDa in size (Willecke et al., 2002) between neighboring cells via passive diffusion through gap junction channels spanning the plasma membranes. GJIC/connexins play an important role in normal development and physiology with a loss of function implicated in several human diseases and syndromes (Krutovskikh and Yamasaki, 2000; Richard, 2001). A multitude of studies involving transgenic mouse strains (knock-outs, knock-ins, tissue-specific deletions) have revealed the importance of connexins in

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developmental and physiological as well as pathological processes (Lo, 1999). Of importance to carcinogenesis, the absence or dysregulation of GJIC in human and animal cells has been associated with decreased growth control and increased tumorigenesis (Mesnil, 2002; Trosko and Ruch, 1998). Many studies have demonstrated a lack of connexin expression or function in human tumors (Mesnil, 2002; Trosko and Ruch, 1998) with induction of GJIC and exogenous expression of connexins in tumor cells restoring at least partial growth control to the tumor cells *in vitro* and/or *in vivo* (King et al., 2000; King et al., 2002; Mehta et al., 1999; Mesnil, 2002; Trosko and Ruch, 1998).

Genetically-engineered mice deficient for the X chromosome-linked Connexin32 gene (Cx32-KO)(Nelles et al., 1996) exhibit dysfunctional physiology as well as pathology (Temme et al., 2001). Following tumor induction with the chemical carcinogen, diethylnitrosamine (DEN), Cx32-KO mice exhibit increased susceptibility to liver tumorigenesis observed as increased tumor incidence and tumor size (Evert et al., 2002; Moennikes et al., 1999; Temme et al., 1997). Similarly, we have shown that exposure to X-ray radiation results in an overall increase in tumor burden in Cx32-KO mice predominantly due to increased liver and lung tumorigenesis (King and Lampe, 2004b). Indeed, we have recently demonstrated that Cx32-KO mice exhibit increased chemical-induced pulmonary tumorigenesis strongly supporting the concept of Cx32 as a lung tumor suppressor (King and Lampe, 2004a).

We noted that a significant portion of radiation-induced Cx32-KO lung tumors also displayed decreased levels of the cell cycle regulator/tumor suppressor p27^{Kip1} (King and Lampe, 2004b). This protein is a member of the CIP/KIP family of cyclin:CDK inhibitors that regulate cell cycle progression at early and late G1/S phase restriction points (Sherr and Roberts, 1999). Decreased p27 protein levels are frequently observed in human cancers and have been used as a clinical prognostic marker indicative of survival in a multitude of human tumors including liver and lung (Lloyd et al., 1999). p27-KO mice demonstrate a paucity of spontaneous tumor development, with the exception of pituitary adenomas. However, studies measuring susceptibility of p27-KO and heterozygous p27 (+/-) mice to radiation and chemical carcinogen-induced neoplasia show that p27 acts as a haploinsufficient tumor suppressor (Fero et al., 1998; Philipp-Staheli et al., 2001). Recently, p27 has been implicated in GJIC-mediated tumor suppressor function. In several studies, expression of exogenous Cx32 or Cx43 into Cx-negative tumor lines resulted in increased p27 levels and partial growth control restoration (Hanna et al., 1999; Koffler et al., 2000). Recently, a role for connexin in cell cycle regulation via interaction with p27-regulating SKP2-associated ubiquitination processes has been suggested (Zhang et al., 2001; Zhang et al., 2003). Therefore, we have generated a mouse strain double-deficient for both Cx32 and p27 (DKO) and evaluated susceptibility to both radiation and chemical-induced, multi-tissue tumorigenesis. Here we compare the altered tumorigenesis, particularly of the lung, as well as tumor biology observed in these studies with respect to genotype and carcinogen induction protocol.

MATERIALS AND METHODS

Mouse Strains and Carcinogen Exposure

Cx32-KO heterozygous mice, originally created in the laboratory of K. Willecke (Nelles et al., 1996) and generously provided by Steven Scherer (Scherer et al., 1998) in a FVB/N background, were intercrossed with p27^{Kip1}-heterozygous mice (C57BL6 background, generously provided by Christopher J. Kemp, FHCRC)(Fero et al., 1996; King et al., 2005) and subsequently inbred for five generations prior to irradiation or DEN treatment. PCR analysis on DNA extracted from tail snips was conducted for mouse genotype determination as previously described (Fero et al., 1996; King and Lampe, 2004a; King and Lampe, 2004b). Radiation experiment: Wild-type mice (38 total), Cx32-KO mice (29 total), p27+/-,Cx32-KO mice (110 total) and double-deficient p27-/-,Cx32-KO mice (DKO, 52 total)

were irradiated at 14 ± 2 days of age with 4 grays (2.6 gray/min) total body exposure using a linear accelerator (King et al., 2005; King and Lampe, 2004b). DEN experiment: Wild-type mice (29 total), Cx32-KO mice (45 total) and DKO mice (35 total) were *i.p.* injected at 12 ± 2 days of age with diethylnitrosamine (DEN, Sigma, St. Louis, MO) at 0.12 μmol DEN/g body weight (King and Lampe, 2004a).

Necropsy, Tissue Processing, Histological Analysis

Mice were sacrificed at the first sign of morbidity or at 17 months of age (Radiation experiment) or 9 months of age (DEN experiment). Necropsies were performed and target organs for the radiation study (liver, lung, pancreas, kidney, adrenal gland, pituitary, small intestine (duodenum), testes, ovary and any/all abnormal or tumorous tissues) or the DEN study (liver and lung) were macroscopically evaluated, formalin fixed, sectioned, stained and microscopically analyzed as previously described (King and Lampe, 2004a; King and Lampe, 2004b). Liver tumor classification as adenoma or carcinoma was based on histological criteria including; mitotic figures, local tissue and blood/lymph vessel invasion, nuclear atypia, anaplastic appearance, trabecular morphology, increased nuclear:cytoplasmic ratio. Lung tumor classification as adenoma or carcinoma was based on criteria as described by the MMHCC (Nikitin et al., 2004) including: mitotic figures, local tissue and blood/lymph vessel invasion, nuclear atypia, anaplastic appearance, increased N:C ratio.

Immunohistochemistry (IHC)

Immunohistochemical detection was conducted as previously described (King et al., 2005; King and Lampe, 2004a; King and Lampe, 2004b). Briefly, tissue sections were deparaffinized, blocked 1 hour at room temperature in a 5% horse serum/PBS solution and detected utilizing a primary antibody against phosphorylated/activated-MAPK (p44/Erk1, p42/Erk2) (1:100, Cell Signaling, Beverly, MA) or pSAPK/JNK (1:100, Cell Signaling, Beverly, MA) in blocking buffer, humidity chamber overnight at 4 degrees or primary antibody against Cyclin D1 (1:50, Neomarkers, Fremont, CA) or β -catenin (1:100, Neomarkers, Fremont, CA) in blocking buffer for one hour at room temperature. Slides were then washed in PBS at room temperature three times for 5 min each followed by one hour incubation with a biotinylated anti-rabbit secondary antibody (1:250, Vector, Burlingame, CA) for 1 hour at room temperature. Following PBS washes, ABC-avidin/biotin conjugate (Vectastain, Vector Labs, Burlingame, CA) was applied for 30 minutes at room temperature with subsequent washes. Signal was detected with addition of diaminobenzidine (DAB) and counterstained with hematoxylin for nuclear detail. MAPK IHC reactivity was typically either extensive or absent and quantified as follows: tumors with less than 2% positive cells were considered MAPK-negative and tumors with more than 30% positive reactive cells were considered to be MAPK-activated. In this study, no tumors were observed with intermediate percentages of reactive cells. Probabilities for incidence, multiplicity and IHC quantification were determined using two-tailed, two sample proportions analysis with significance at $p \leq 0.05$.

Analysis of Liver Tumor Ha-ras and β -catenin Mutational Status

As previously described (King et al., 2005), DNA was extracted from paraffin-embedded liver tumor tissue for determination of the mutational status of Ha-*ras* (codon 61) and β -catenin (exons 2 and 3) using the EX-WAX DNA extraction kit (Chemicon, Temecula, CA). DNA was then precipitated and purified for subsequent PCR amplification with Ha-*ras* and β -catenin primers followed by DNA sequencing.

RESULTS

Cx32/p27-DKO Mice Exhibit General Characteristics of p27-KO Mice with Altered Tumorigenesis

Several studies have utilized mouse strains with targeted-genetic knockout of Cx32 or p27 genes to evaluate the individual impact of these two tumor suppressors on tumorigenesis in a wide spectrum of tissues in response to both chemical carcinogens (Evert et al., 2002; Fero et al., 1998; King and Lampe, 2004a; Moennikes et al., 1999; Temme et al., 1997) as well as radiation (King and Lampe, 2004b). As previously detailed (King et al., 2005), we intercrossed Cx32-KO and p27-KO mice generating a mouse strain deficient for both Cx32 and p27 (DKO) where mice were viable and demonstrated many general characteristics previously described for p27-KO mice such as female infertility, increased body weight and spontaneous pituitary tumorigenesis (Fero et al., 1996). We have previously reported results from radiation-induced tumorigenesis studies where we observed increased overall tumor susceptibility, particularly of the liver and lung, in Cx32-KO mice (69%) compared to Wild-type mice (42%, $p=0.007$) (King and Lampe, 2004b). Irradiation of DKO mice resulted in an even greater overall increase in tumorigenesis (87%, $p<0.005$ compared to Wild-type and $p=0.025$ compared to Cx32-KO) but was surprisingly associated with a decreased liver and lung tumor incidence suggesting Cx32/p27 pathway interaction (King et al., 2005). Chemical carcinogen induction (DEN) studies with Cx32-KO mice have repeatedly demonstrated increased liver tumorigenesis (Evert et al., 2002; Moennikes et al., 1999; Temme et al., 1997) and recently we showed increased lung tumor susceptibility (King and Lampe, 2004a). These studies underscore the importance of Cx32/GJIC in tumor suppression in multiple tissue types and in response to various carcinogen types. Here we describe a detailed comparison of variation in tumor formation where Wild-type, Cx32-KO and DKO mice were treated with different inductive agents, thereby shedding light on the inherent tissue-specific variation in carcinogen susceptibility with respect to genotype, tumor incidence and multiplicity as well as associated tumor biology, here observed as dramatically altered MAPK activation.

Liver tumorigenesis in KO mice

Incidence—We have previously observed dramatically increased liver tumor incidence in irradiated Cx32-KO mice (16% to 46%)(King and Lampe, 2004b) with an even more striking increase in tumor incidence in DEN-treated mice (38% to 85%)(Figure 1A, Table 1). These results are in support of previously published DEN-induced Cx32-KO liver tumorigenesis studies and strongly implicate Cx32 as a tumor suppressor in mouse liver (Evert et al., 2002; Temme et al., 1997). p27 expression is employed as a clinical indicator of survival in human hepatocarcinogenesis and is implicated in mouse liver tumorigenesis (Lloyd et al., 1999; Philipp-Staheli et al., 2001). Surprisingly, we observed that additional loss of p27 in a Cx32-KO background did not increase radiation-induced liver tumor frequency as might have been expected (Figure 1A, Table 1). In fact, additional loss of p27 resulted in a significant decrease in tumor incidence (13%) compared to Cx32-KO mice (46%, $p=0.002$) (King et al., 2005). Here we present different results with DEN induction where we observed that loss of both Cx32 and p27 resulted in essentially no change in liver tumor incidence (86%, Figure 1A) compared to Cx32-KO only (85%). Results from the radiation study suggest that p27 may play a tumor promoter role in mouse liver tumorigenesis, at least in a Cx32-deficient background. Clearly this is not supported by the DEN study results opening the possibility that particular pathways uniquely activated by specific carcinogens influence Cx32/p27 pathway interactions and coordinated functions. However, in total these results are in accord with a hypothesis that the absence of Cx32 and/or Cx32-mediated GJIC may alter or diminish p27 tumor suppressor function which is apparently subject to carcinogen specificity.

Multiplicity—In addition to increased tumor incidence, the average number of liver tumors/tumor-bearing mice (multiplicity) was also increased in irradiated Cx32-KO mice (1.9 tumors/mouse) compared to Wild-type mice (1.5 tumors/mouse, Table 2). As DEN is a more efficient liver carcinogen, a more dramatic increase in multiplicity was observed in DEN-treated Cx32-KO mice (17 tumors/mouse) compared to Wild-type mice (3 tumors/mouse). Interestingly, loss of p27 increased the average liver tumor multiplicity in DEN-treated DKO mice (21 tumors/mouse) but resulted in a decrease in irradiated DKO mice (1.3 tumors/mouse). These results suggest a possible disparity in tumor multiplicity that corresponds to both carcinogen and p27 genetic background.

Lung tumorigenesis in KO mice

Incidence—As previously published (King and Lampe, 2004b), lung tumor incidence is modestly increased in irradiated Cx32-KO mice (21% to 31%) supported further by a significantly increased incidence in DEN-treated mice (24% to 62%)(Table 3, Figure 1B)(King and Lampe, 2004a). Clearly these results implicate Cx32 as a tumor suppressor in mouse lung. Similarly, deletion of the tumor suppressor p27 in a mouse strain has been reported to significantly increase lung tumorigenesis in response to carcinogen induction (Fero et al., 1998). However, in a combined DKO mouse strain, we have shown that additional loss of p27 in a Cx32-KO background did not increase radiation-induced lung tumor incidence (Table 3) (King et al., 2005). In fact, similar to liver results, additional loss of p27 actually decreased tumor incidence (12%) compared to Cx32-KO mice (31%, $p=0.061$). Here, as was the case with liver, we show that additional loss of p27 resulted in a slight but not statistically significant increase in lung tumor incidence in DEN-treated DKO mice (74%, Figure 1B) compared to Cx32-KO only (62%, $p=0.247$) in DEN-treated mice. These results strongly implicate genotype background in modulation of the differential action of carcinogens in lung tumorigenesis in these mouse models.

Multiplicity—In addition to increased tumor incidence, the average number of lung tumors/tumor-bearing mice (multiplicity) was also increased in irradiated Cx32-KO mice (1.5 tumor/mouse) compared to Wild-type mice (1.0 tumor/mouse Table 2). A similar increase was observed in DEN-treated Cx32-KO mice (1.6 tumor/mouse) compared to Wild-type mice (1.1 tumor/mouse). Additional loss of p27 dramatically increased the lung tumor multiplicity in both irradiated DKO mice (2.8 tumors/mouse) and DEN-treated DKO mice (2.0 tumors/mouse).

Progression—We have previously reported that irradiated Cx32-KO mice exhibit an increased percentage of lung carcinomas (40%) compared to irradiated Wild-type mice (12%). This increase in tumor progression in the absence of Cx32 was maintained in the DKO mice (53% carcinoma, $p=0.014$ compared to Wild-type) implying that either the Cx32 genotype alone dictates tumor progression profile or that the two pathways overlap in some manner with additional loss of p27 in a Cx32-KO background therefore having a lesser impact. In mice treated with DEN, we observed a similar increase in tumor progression in Cx32-KO mice (29% carcinoma) compared to Wild-type mice (12%). As in the radiation study, this carcinoma percentage was similar albeit slightly higher in the DKO mice as well (37%). The differential sensitivity of DKO mouse lung to radiation and DEN-induced tumorigenesis again suggests a distinct set of altered molecular pathways in response to the different carcinogens.

Analysis of Potential Oncogenic Alterations in Liver and Lung Tumors

As liver and lung were the predominant tissue types affected in irradiated Cx32-KO and DKO mice and in an attempt to determine genotype-specific molecular mechanisms of tumorigenesis in this model, we employed IHC techniques to evaluate alterations in signaling pathways that represent common targets for oncogenesis in mice.

In liver tumors, we analyzed β -catenin and Cyclin D1 expression levels as activation, mutation, over-expression and/or stabilization of these cell cycle regulators has been repeatedly observed in liver neoplasia (representative results shown in Figure 2A). In both liver and lung tumors, β -catenin staining appeared to be increased compared to surrounding normal tissue but was predominantly plasma membrane localized and did not appear to be significantly increased in the nucleus (liver example in Figure 2A). These increased β -catenin levels did not appear to correlate with genotype or any of the other protein markers analyzed in this study. As β -catenin mutations are often associated with mouse liver tumorigenesis, we sequenced β -catenin exons 2 and 3 from the same liver tumors detailed above (King et al., 2005). We found no base substitutions in the commonly mutated codons (32, 33, 37, 41) or any insertions or deletions in this same area. This suggests an oncogenic mechanism independent from mutation in these tumors that results in increased β -catenin protein levels. Similar increases in β -catenin protein levels using IHC techniques were detected in DEN-induced liver tumors. Likewise, Cyclin D1 expression was increased in a predominance of liver tumors but did not correlate with genotype or other markers evaluated (example Figure 2A).

In radiation-induced lung tumors, in addition to β -catenin levels, we evaluated SAPK/JNK activation (representative result shown in Figure 2A) as over-stimulation of these signaling pathways are often observed in pulmonary neoplasia usually resulting from mutations within *K-ras*. In lung, no JNK activation was observed perhaps indicating that *K-ras* mutations were not predominant in these particular tumors. Indeed with respect to liver, we have previously presented data obtained from sequencing a majority of liver tumors from each genotype group for *Ha-ras* (codon-61) missense mutations and discovered no significant difference between genotypes (King et al., 2005). It is worth noting that there are other known codons that are mutated at a high frequency in the *Ha-ras* gene following chemical carcinogen treatments that were not evaluated in this study. In any case, this sequence data indicates that activation of *Ha-ras* via mutation is not a common mechanism of oncogenic alteration in these tumor groups.

MAPK-activation in KO Liver and Lung Tumors

MAPK signaling pathways are frequently hyperactivated in both mouse liver and lung tumors (Hilger et al., 2002; Ito et al., 1998). This is often due to oncogenic alterations in growth factor receptor pathways commonly via mutations within *Ha-ras/K-ras* coding sequences resulting in constitutive protein activation. We, therefore, analyzed radiation-induced liver and lung tumors and DEN-induced lung tumors for MAPK activation/phosphorylation utilizing IHC techniques and a phosphospecific antibody that reacts solely with activated/phosphorylated-MAPK.

Liver tumors

We have previously reported that Cx32-KO mice exhibit an increased percentage of activated-MAPK liver tumors following radiation (King and Lampe, 2004b). We have also noted that additional loss of p27 in a Cx32-KO background resulted in a significantly decreased percent of MAPK-activated liver tumors (33%) in contrast to the increased activation observed in Cx32-KO liver tumors (75%, $p=0.027$) (King et al., 2005). It is evident that MAPK activation was not strictly correlated with more advanced tumor types (carcinoma) as MAPK-activated adenomas were also significantly increased in Cx32-KO and DKO mice compared to Wild-type mice.

Lung tumors

Radiation Study—We have previously reported that IHC analysis of radiation-induced lung tumors revealed a significantly increased percentage of MAPK-activated lung tumors from Cx32-KO mice (60%) compared to those from Wild-type mice (13%, $p=0.016$, Table 4, Figures 2B and 3) (King and Lampe, 2004b). Activation of MAPK pathways often correlates with

advanced tumor progression in mouse lung (Ramakrishna et al., 2002; Yano et al., 1999; Yao et al., 2003); however, as the only carcinoma detected in the Wild-type group exhibited no detectable MAPK-activation, this general hypothesis cannot explain the observed increase in MAPK-activation in Cx32-KO lung tumors. Interestingly, additional total deletion of p27 resulted in the loss of MAPK-activated lung tumors in DKO mice ($p < 0.001$ compared to Cx32-KO, Table 4, Figures 2B and 3) (King et al., 2005). These results suggest that loss of Cx32 may predispose lung tumors to progress toward carcinoma, perhaps via increased MAPK pathway activation influenced by p27. However, in the absence of p27, tumors are influenced or preferentially selected to form tumors independent from significant MAPK pathway activation.

DEN study—In support of results from the radiation study described above, we have previously shown a dramatic increase in the percentage of MAPK-activated lung tumors in DEN-induced Cx32-KO mice (27%) compared to Wild-type mice (0%, $p < 0.001$, Table 5, Figures 2B and 3) (King and Lampe, 2004a). Here, we present data showing that additional loss of p27 in a Cx32-KO background results in a decrease in MAPK-activated lung tumors (18%) in DEN-treated DKO mice compared to Cx32-KO mice (27%, Table 5, Figures 2B and 3). Although not statistically significant, this is similar to the trend observed previously in irradiated DKO lung tumors (Table 4). These results suggest that in lung, a Cx32-deficient background contributes to p27 pathway modulation. Specifically, that p27 tumor suppression is invalidated to some extent in the absence of Cx32/GJIC culminating in an increased susceptibility to two distinct types of carcinogenic insult (radiation and chemical).

A comparison of the effects of these two different carcinogens in lung tumorigenesis in these deficient mouse models underscores variation not only in particular carcinogen efficiency, but also demonstrates the drastic difference in susceptibility to both carcinogens between Wild-type and Cx32-KO or DKO mice (Table 6). This is manifest as no statistical difference in the percent of tumors with activated MAPK when comparing DEN with radiation in Wild-type groups but significant differences in the percent of MAPK positive tumors in Cx32-KO and DKO groups when comparing the two carcinogens.

Discussion

Here, we compared the interactive roles of two tumor suppressor proteins by utilizing Cx32-KO and Cx32/p27-DKO mouse strains to demonstrate individual and combined gene influence on radiation and chemical-induced tumorigenesis. When compared to Wild-type mice or each other, both Cx32-KO and DKO mouse mice demonstrated differential sensitivity to radiation-induced tumorigenesis involving several organ systems consistent with their known tissue-specific tumor suppressor functions. This was predominantly due to tumorigenesis of the liver (Evert et al., 2002; King and Lampe, 2004b; Moennikes et al., 1999; Temme et al., 1997) and lung (King and Lampe, 2004a; King and Lampe, 2004b) in a manner dependent on Cx32 expression as well as tissues that demonstrate a strong dependence on p27 tumor suppressor function as seen in previous studies using p27-KO mice (intestine, pituitary, adrenal and tissue of hematopoietic origin (thymic lymphoma)) (Fero et al., 1998; Philipp-Staheli et al., 2001).

Unexpectedly, we have observed tissue-specific alterations in p27 tumor suppressor function dependent on Cx32 background. Additional loss of p27 in Cx32-deficient liver resulted in a statistically significant decrease in tumorigenesis following radiation-induction. While not statistically significant, we observed a similar trend in radiation-induced lung tumorigenesis in these mice. Even though p27 is implicated as a tumor suppressor in these two tissues (Fero et al., 1998), additional loss of p27 did not result in increased liver and lung tumor incidence in a Cx32-KO background but actually resulted in decreased liver tumor incidence and multiplicity. These results are unexpected as several studies have demonstrated increased tumor incidence and/or increased tumor progression using mouse models with combined-

deficiency in p27 and various other tumor suppressors or oncogenes (Philipp-Staheli et al., 2004; Philipp-Staheli et al., 2002). However, in support of a crucial positive role for p27 in the process of tumorigenesis, several studies have demonstrated a tissue-specific dependence on p27 for mouse mammary tumorigenesis (Muraoka et al., 2001).

An increase in lung tumor progression (increased carcinoma to adenoma ratio) was also observed in Cx32-KO mice but was not further altered in DKO mice. This contrasts with the observed liver tumorigenesis where losses of Cx32 or both Cx32 and p27 affected tumor incidence without altering tumor progression. We and others have noted increased BrdU incorporation in Cx32-KO liver and lung tissue indicating increased proliferation in these non-tumorous tissues may partially explain the increased susceptibility to carcinogen treatments (King and Lampe, 2004a; Temme et al., 1997).

Interestingly, while the general trend of increased tumor incidence in Cx32-KO mice holds for both DEN and radiation induction, a comparison of the results from these two carcinogen studies offers possible insight into molecular mechanisms related to different modes of initiation. In Wild-type mice, no significant difference in lung tumor incidence (Table 3) or MAPK-activation (Table 6) is observed when comparing radiation and chemical carcinogen treatments. In contrast, Cx32-KO mice demonstrate a significantly different tumor incidence depending on the induction method employed (Table 3). At first glance this may not seem surprising as these induction modes are quite distinct. However, no such disparity in susceptibility was observed with the Wild-type mice suggesting influence of genotype. Additionally, there appears to be differences in the molecular alterations associated with both carcinogens in Cx32-KO mice. For example, there is a significant difference in the percentage of MAPK-active lung tumors in the chemical and radiation studies (Table 6). Radiation induction resulted in a less dramatic increase in incidence but more of these lung tumors contained activated-MAPK pathways. Treatment with DEN resulted in a significant increase in lung tumor incidence; however, fewer tumors were MAPK-activated. Analysis of these differences may allow better elucidation of the molecular alterations associated with Cx32-deficiency that result in an increased proclivity toward developing liver and lung tumors. These comparisons suggest that Cx32 pathways may interact with several other oncogenic pathways that rely on MAPK-dependent as well as independent effectors. Further description of the molecular character of liver and lung tumors from these Cx32-KO mice, as well as comparison to still other initiating compounds (such as urethane or ethylnitrosourea), may allow further discovery of Cx32/GJIC-influenced or interacting pathways responsible for the observed increased tumorigenesis. Again, whether these tumor susceptibility/tumor biology differences represent disparity in mutation-related cell initiation or tumor evolution/selection remains to be determined.

Altogether, these results from radiation and DEN liver and lung tumor induction studies indicate that loss of Cx32 results in increased liver and lung tumor incidence with both carcinogens. In contrast, additional loss of p27 can decrease (radiation) or increase (DEN) tumor incidence in a Cx32-KO context depending on the carcinogen. In both cases Cx32-deficiency did result in increased multiplicity and increased progression of liver and lung tumors (observed as an increased carcinoma:adenoma ratio) that is further elevated by the absence of p27. These results may indicate that radiation and DEN act via distinct molecular mechanisms and may therefore interact differentially with Cx32/GJIC pathways. This is evident as increased MAPK-activation in Cx32-KO mice where additional loss of p27 differentially decreases (radiation) or increases (DEN) this phenotype.

Aberrant connexin expression and dysregulated GJIC is correlated with diminished growth control and increased neoplastic potential (Mesnil, 2002; Trosko and Ruch, 1998), however precise mechanisms by which any specific connexin exerts tumor suppressor activity remains

speculative. For example, multiple studies have demonstrated elevated susceptibility to liver tumorigenesis following treatment with the chemical carcinogen DEN (Evert et al., 2002; Moennikes et al., 1999; Temme et al., 1997), however no molecular/mutational mechanism of action has been determined. Here we showed preferential origination/selection of liver and lung tumors exhibiting increased MAPK activation from Cx32-KO mice. Interestingly, MAPK has been shown to directly modulate connexin/GJIC life cycle and activity via Cx43 phosphorylation both *in vitro* and *in vivo* (Defamie et al., 2003; Upham et al., 2003; Warn-Cramer et al., 1998; Warn-Cramer et al., 1996).

Our data suggests the possibility that in a Cx32-deficient environment, tumors with active MAPK pathways may possess a selective growth advantage compared to tumors lacking higher MAPK activation. In this model, p27 would in some capacity further support increased numbers of MAPK-active tumors to develop/progress or in contrast result in an inhibition of MAPK-inactive tumor development. As p27 deletion results in decreased tumor numbers as well as decreased MAPK-active tumors, it is possible that the observed decrease in tumors is partially due to the specific loss of MAPK-active tumors.

Increased murine hepatocarcinogenesis is often linked with MAPK pathway activation resulting from Ha-*ras* mutation (Lumniczky et al., 1998; Schwarz et al., 2003). In these studies, we did not detect any correlation between Cx32/p27 genotype and the frequency of liver tumors harboring activating Ha-*ras* codon-61 mutations nor did we detect any β -catenin exon 2 or 3 codon mutations, insertions or deletions. Additionally, a comparison of each individual tumor using both immunohistochemistry and sequencing yielded no direct correlation between Ha-*ras* mutation and MAPK-activated status in the liver tumors examined (not shown). In lung tumors, we noted no SAPK/JNK kinase activation suggesting that activation of K-*ras* via mutation is not necessarily a common mechanism of lung tumorigenesis in these mouse models.

As a majority of tissues demonstrate expression of multiple connexin types, the possibility remains that other connexin family members are the important effectors of tumorigenesis or that several connexins share tumor suppressor function redundancy. Since we have detected several tissues that demonstrate a particular dependence on Cx32 and/or p27 for tumor suppressor function, we can employ more directed carcinogens, for example urethane, to further evaluate the separate and interactive roles of Cx32 and p27 in mouse carcinogenesis. To further evaluate and define the molecular mechanisms by which connexin/GJIC and p27-associated pathways interact and influence multi-organ tumorigenesis, we will continue to investigate the role of Cx32-mediated GJIC and p27 function in regulation of MAPK signaling pathways.

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Figure 1.

Comparison of liver and lung tumor incidence in Wild-type, Cx32-KO and Cx32/p27-DKO mice exposed to either radiation (light bars) or a chemical carcinogen (DEN, dark bars). Note the biphasic quality of incidence in the radiation study compared to the linearity of the DEN study.

Figure 2. Immunohistochemical detection of liver and lung tumors for tumorigenesis related proteins. A: liver, cyclin D1 (left panel); liver, β -catenin (center panel); lung, activated-SAPK/JNK (right panel). B: Lung tumors detected using a phosphospecific antibody reactive against only activated/phosphorylated MAPK from DEN-induced (top panels) or radiation-induced (bottom panels) Wild-type (left panels), Cx32-KO (center panels) and Cx32/p27-DKO (right panels) mice. Bar=50 μ m.

Figure 3.

Comparison of the percentage of lung tumors showing MAPK-activation from radiation-induced (light bars) or DEN-induced (dark bars) Wild-type, Cx32-KO and Cx32/p27-DKO mice.

Table 1

Carcinogen comparison of liver tumor incidence.

Group	DEN	RAD	p value (DEN/RAD)
Wild-type	38 % (11/29)	16 % (6/38)	0.035
Cx32-KO	85 % (39/46)	46 % (12/26)	<0.0001
Cx32/p27 ^{+/-}	86 % (93/108)	25 % (28/110)	<0.0001
Cx32/p27-DKO	86 % (30/35)	13 % (7/52)	<0.0001
p value = WT:KO	<0.001	0.009	-
P value = KO:DKO	0.9	0.002	-

Table 2

Tumor multiplicity (# tumors/tumor+mice).

Group	<u>Liver</u>		<u>Lung</u>	
	<u>DEN</u>	<u>RAD</u>	<u>DEN</u>	<u>RAD</u>
Wild-type	3	1.5	1.1	1.0
Cx32-KO	17	1.9	1.6	1.5
Cx32/p27-DKO	21	1.3	2.0	2.8

Table 3

Carcinogen comparison of lung tumor incidence.

Group	DEN	RAD	p value (DEN/RAD)
Wild-type	24 % (7/29)	21 % (8/38)	0.771
Cx32-KO	62 % (28/45)	31 % (8/26)	0.008
Cx32/p27-DKO	74 % (26/35)	12 % (6/52)	<0.001
p value = WT:KO	0.001	0.373	-
p value = KO:DKO	0.247	0.061	-

Table 4
Radiation-induced lung tumors/MAPK activation in KO mice.

<u>Group</u>	<u>Tumor Incidence</u>	<u>Total Lung Tumors</u>		<u>Total</u>	<u>pMAPK</u>	
		<u>Adenoma</u>	<u>Carcinoma</u>		<u>Adenoma</u>	<u>Carcinoma</u>
Wild-type	21 % (8/38)	88 % (7/8)	13 % (1/8)	13 % (1/8)	14 % (1/7)	0 % (0/1)
Cx32-KO	31 % (8/26)	58 % (7/12)	42 % (5/12)	60 % (6/10)	40 % (2/5)	80 % (4/5)
Cx32/p27 DKO	12 % (6/52)	47 % (8/17)	53 % (9/17)	0 % (0/14)	0 % (0/6)	0 % (0/8)
p value = WT-KO	0.373	-	-	0.016	-	-
p value = KO-DKO	0.061	-	-	<0.001	-	-

Table 5
DEN-induced lung tumors/MAPK activation in KO mice.

<u>Group</u>	<u>Tumor Incidence</u>	<u>Total Lung Tumors</u>		<u>Total</u>	<u>pMAPK</u>	
		<u>Adenoma</u>	<u>Carcinoma</u>		<u>Adenoma</u>	<u>Carcinoma</u>
Wild-type	24 % (7/29)	88 % (7/8)	13 % (1/8)	0 % (0/8)	0 % (0/7)	0 % (0/1)
Cx32-KO	62 % (28/45)	71 % (32/45)	29 % (13/45)	27 % (12/45)	42 % (5/12)	58 % (7/12)
Cx32/p27 DKO	74 % (26/35)	63 % (32/51)	37 % (19/51)	18 % (9/50)	10 % (3/31)	32 % (6/19)
p value = WT-KO	<0.001	-	-	<0.001	-	-
p value = KO-DKO	0.247	-	-	0.293	-	-

Table 6

Carcinogen comparison of MAPK activation in lung tumors.

MAPK-Active Tumors	DEN	RAD	p value
Wild-type	0 % (0/8)	13 % (1/8)	0.274
Cx32-KO	27 % (12/45)	60 % (6/10)	0.050
Cx32/p27-DKO	18 % (9/50)	0 % (0/14)	0.001