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Neural Tube Development Requires the Cooperation of p53- and Gadd45a-Associated Pathways

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BACKGROUND: Numerous genetically engineered mouse models for neural tube defects (NTDs) exist, and some of the implicated proteins are functionally related. For example, the growth arrest and DNA damage-inducible protein Gadd45a and tumor suppressor p53 are functionally similar, and both are involved in neural tube development (*Gadd45a*- and *Trp53*-null embryos show low levels of exencephaly). To assess their roles in neural tube development, we generated double-null mice from *Gadd45a*- and *Trp53*-null mice, as well as from *cyclin-dependent kinase inhibitor* (*Cdkn1a*) (p21)-null and *xeroderma pigmentosum group C* (*XPC*)-null mice that do not show spontaneous exencephaly. **METHODS:** *Gadd45a*-, *Trp53*-, *Cdkn1a*-, and *XPC*-null mice were crossed to generate several double-null mouse models. Embryos (embryonic day [ED] 16–18) from the single- and double-null crosses were scored for NTDs. **RESULTS:** Deletion of both *Gadd45a* and *Trp53* in mice increased exencephaly frequencies compared to the deletion of either single gene (34.0% in *Gadd45a/Trp53*-null compared to 8.4% and 9.1% in the *Gadd45a*- and *Trp53*-null embryos, respectively). Furthermore, although deletion of another p53-regulated gene, *Cdkn1a*, is not associated with exencephaly, in conjunction with *Gadd45a* deletion, the exencephaly frequencies are increased (30.5% in the *Gadd45a/Cdkn1a*-null embryos) and are similar to those in the *Gadd45a/Trp53*-null embryos. Although *XPC* deletion increased exencephaly frequencies in *Trp53*-null embryos, *XPC* deletion did not increase the exencephaly frequencies in *Gadd45a*-null embryos. **CONCLUSIONS:** The increased genetic liability to exencephaly in the *Gadd45a/Trp53*- and *Gadd45a/Cdkn1a*-null embryos may be related to the disruption of multiple cellular pathways associated with Gadd45a and p53. *Birth Defects Research (Part A) 76:129–132, 2006.* © 2006 Wiley-Liss, Inc.†

Key words: Gadd45a; p53; p21; XPC; exencephaly

INTRODUCTION

Neural tube defects (NTDs) are the second most frequent form of human birth defects (reviewed in Copp et al., 2003). In developing embryos, one can experimentally induce NTDs during the time of neural tube closure by administering a wide range of treatments to the pregnant female, including exposure to ionizing radiation or cyclophosphamide, inducing hyperthermia, or feeding the pregnant female a vitamin-deficient or -excessive diet (Senyszyn and Rugh, 1969; Seller et al., 1983; Chernoff et al., 1989; Fleming and Copp, 1998; Yitzhakie et al., 1999). Because NTDs can be induced by such diverse means, it is clear that neural tube closure is a complex developmental event that involves multiple factors and their associated pathways.

The multifactorial nature of NTDs is also exemplified by the >80 mouse models of NTDs (reviewed in Juriloff and Harris, 2000 and Copp et al., 2003). A review of these mutant mice implicates pathways involved in apoptosis, cell-cycle arrest, DNA repair, and genome integrity main-

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tenance as critical events during neural tube development. For example, both Gadd45a and p53 are known to function in each of these pathways, and their role in neural tube development is highlighted by the fact that ~10% of embryos from *Gadd45a*- and *Trp53*-null mice have exencephaly (Sah et al., 1995; Hollander et al., 1999). However, despite the ubiquitous roles played by Gadd45a and p53, it has been difficult to elucidate their critical function(s) during neural tube development.

The risk of developing a multifactorial threshold disorder, such as NTDs, increases with the number of mutant genes contributed by each parent. For example, *Trp53*-null mice exhibit exencephaly, but when *Trp53*-null mice were crossed with *XPC*-null mice to generate *Trp53/XPC*-null mice, the observed frequency of exencephaly was increased from 0% and 8.4% in the *XPC*- and *Trp53*-null embryos, respectively, to 29.6% in the *Trp53/XPC*-null embryos (Sah et al., 1995; Cheo et al., 1996). Although undetected background strain genes may contribute to the observed phenotype, the increased genetic liability to exencephaly is likely due to the reduced activity of XPC- and p53-associated pathways. Because XPC is primarily a DNA repair factor, it is likely that this role is especially critical in the absence of other factors, such as p53, whose functions span many key cellular pathways.

Designing experiments to elucidate the function of Gadd45a and p53 during neural tube development is complicated by the fact that each protein has been attributed to a variety of functions in the cell. Both are known to be important factors in maintaining genome stability, regulating apoptosis induction, G1 cell-cycle checkpoint control through p21, and global genomic repair (reviewed in Donehower et al., 1997; Smith et al., 1996; Kearsey et al., 1995; Hollander et al., 1999; Smith et al., 2000; Hildesheim et al., 2002; Bulavin et al., 2003). However, if Gadd45a and p53 are directly involved in a shared pathway during neural tube development, such as G1 checkpoint control through p21, the liability to exencephaly would not be expected to differ markedly between *Gadd45a*-, *Trp53*-, and *Gadd45a/Trp53*-null genotypes. However, if Gadd45a and p53 contribute to neural tube closure through different pathways, the liability to exencephaly would be expected to increase.

Because of the close association and functional interactions between Gadd45a and p53, we hypothesized that the similar frequencies of exencephaly observed in mouse strains lacking either *Trp53* or *Gadd45a* might be due to the disruption of a shared pathway. To study this issue, we crossed mice lacking *Gadd45a*, *Cdkn1a*, *Trp53*, or *XPC* to generate several double-null models. Surprisingly, *Gadd45a/Trp53*- and *Gadd45a/Cdkn1a*-null mice developed exencephaly of equal severity but at a much higher frequency than mice with a single gene deletion. Alternatively, deletion of both *Gadd45a* and *XPC* did not promote increased exencephaly frequency. In conjunction with previously reported *XPC/Trp53*-null mice studies, the phenotypes of the double-null mice suggest that XPC and Gadd45a may operate separately from p53 and p21 during neural tube development and illustrate the cooperative effects of multiple pathways (e.g., genome stability, apoptosis, cell-cycle arrest, and/or DNA repair) during neural tube development.

MATERIALS AND METHODS

Breeding Strategies

Gadd45a-, *Trp53*-, *Cdkn1a*-, and *XPC*-null mice have been described previously (Donehower et al., 1992; Deng et al., 1995; Cheo et al., 1996; Hollander et al., 1999). The $Gadd45a^{+/-}/Trp53^{+/-}$ double-heterozygous mice used for breeding were generated by mating a *Gadd45a*-null female with a *Trp53*-null male. All *Gadd45a/Trp53*- and *Trp53*-null animals in this study were derived from offspring of these original founders, and thus both genotypes had the same mixed genetic background (SJL, 129sv, and C57BL/6). For generation of the *Trp53*- and *Gadd45a/Trp53*-null mice, breeders consisted of $Gadd45a^{+/-}/Trp53^{-/-}$, $Gadd45a^{-/-}/Trp53^{-/-}$, or $Gadd45a^{-/-}/Trp53^{+/-}$ females and males. This approach was taken to increase the odds of obtaining *Gadd45a/Trp53*-null progeny in spite of frequent birthing complications (dystocia) in *Gadd45a/Trp53*-null females. *Gadd45a*-, *XPC*-, and *Cdkn1a*-null mice were maintained as a mixed background of 75% 129sv and 25% C57BL/6, with no subsequent back-crossing. The *Gadd45a/Cdkn1a*- and *Gadd45a/XPC*-null mice were 75% 129sv and 25% C57BL/6 and were maintained by crossing double-null males and females. The mice were housed in Plexiglas cages and given autoclaved NIH 31 diet and water ad libitum. The animal facilities of the National Institutes of Health (NIH) are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), and all experiments were done under an approved NIH animal study protocol.

NTD Observations and Scoring

NTDs typically are not observed in newborns, due to lethality and subsequent cannibalization. Therefore, embryos at embryonic day (ED) 16–18 were taken from pregnant females. The embryos were examined for NTDs, including exencephaly (caudal failure), spina bifida (rostral failure), and curly tail. Genotypes were determined by PCR of tail lysates from the embryos.

Statistics

Statistical analysis was performed using the Prism Software package (GraphPad Software, San Diego, CA). Fisher's exact test was performed among and between the single- and double-null groups for exencephaly analysis. $P < .05$ was considered statistically significant.

RESULTS

The frequency of exencephaly in *Gadd45a*-null mice was similar to that in *Trp53*-null embryos and to published data for *Trp53*-null mice (Fig. 1A, Table 1) (Sah et al., 1995). However, deletion of both *Gadd45a* and *Trp53* led to a much higher frequency of exencephaly (34.0% in *Gadd45a/Trp53*-null, $P < .05$ when *Gadd45a/Trp53*-null embryos were compared with either *Trp53*- or *Gadd45a*-null embryos) than when either gene alone was deleted (Table 1). In addition to exencephaly, curly tail was rarely observed in the *Gadd45a/Trp53*-null mice (3.0%).

The p53-regulated protein p21 is the major p53 effector for the G1 checkpoint (Deng et al., 1995; Sheikh et al., 1995). We generated *Gadd45a/Cdkn1a*-null mice to determine the effect of the loss of 2 p53-regulated genes on neural tube development. *Cdkn1a*-null mice have not been reported to exhibit exencephaly, and we found no affected pups in 7

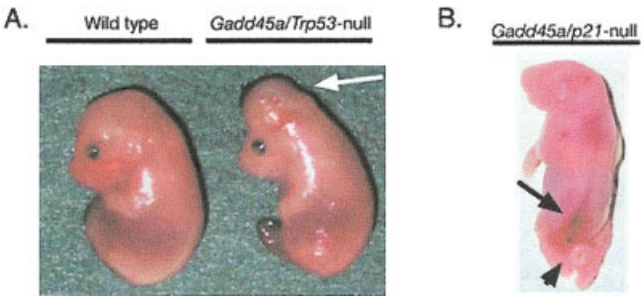

Figure 1. Deletion of *Gadd45a* in conjunction with either *Trp53* or *Cdkn1a* leads to NTDs. **A:** Comparison of a wild-type embryo and a *Gadd45a/Trp53*-null embryo with exencephaly (white arrow). **B:** A *Gadd45a/Cdkn1a*-null newborn mouse with spina bifida (black arrow) and curly tail (black arrowhead). *Newborn mice were not included in this data set, but this picture is included to illustrate spina bifida seen in the *Gadd45a/Cdkn1a*-null progeny.

litters (53 total embryos) from *Cdkn1a*-null crosses (Table 1). However, in crosses between *Gadd45a/Cdkn1a*-null mice, the frequency of exencephaly was 30.5% ($P < .05$ when *Gadd45a/Cdkn1a*-null embryos were compared with *Gadd45a*-null embryos), and spina bifida was observed at low frequencies (2.9%) (Fig. 1B).

Because *Gadd45a* deletion may augment the *Trp53*-null exencephaly phenotype in a manner similar to deletion of *XPC*, we generated *Gadd45a/XPC*-null mice to assess their cooperation in neural tube development. We found no apparent developmental defects in *XPC*-null mice, and the *Gadd45a/XPC*-null mice did not have a greater frequency of exencephaly than the *Gadd45a*-null mice (Table 1).

DISCUSSION

Gadd45a and p53 are known to share similar cellular functions, in particular the maintenance of genome stability and S-phase checkpoint control (Hollander et al., 1999, 2005). Although additional cellular roles in apoptosis and cell-cycle control have been proposed for both Gadd45a and p53, specific similarities have not been delineated. On the basis of these functional associations and the low prevalence of exencephaly in *Gadd45a*- and *Trp53*-null embryos, we hypothesized that Gadd45a and p53 proteins might contribute to neural tube development through a shared pathway. However, the fact that *Gadd45a* deletion significantly augmented exencephaly liability when *Trp53* was also deleted indicated that these proteins might be contributing to neural tube development through different pathways. Were this not the case, both *Gadd45a/Trp53*-null mice and embryos would be expected to have the same genetic liability to exencephaly to that observed in *Trp53*- or *Gadd45a*-null mice. In support of this conclusion, deletion of the G1 checkpoint protein p21, which is transcriptionally activated by p53, is not sufficient to cause exencephaly (possibly due to redundancies originating from p53), but combined with *Gadd45a* deletion, the genetic liability to exencephaly is significantly augmented (Table 1). This suggests that deletion of either *Cdkn1a* or *Trp53* increases the genetic liability to exencephaly in the *Gadd45a*-null embryo through a similar pathway. Of interest, *XPC/Trp53*-null mice develop exencephaly at a much higher frequency compared to mice that lack either gene alone (Cheo et al., 1996). However, *Gadd45a/XPC*-null genetic liability was not increased, which suggests that these proteins may share a similar role in neural tube development that is independent of p53 and p21 function.

The functional roles of Gadd45a, p21, p53, and XPC are fairly well characterized and, as shown by our data, this group of genes serves at least 4 of the major functions required for normal neural tube development (genome stability, G1 checkpoint, apoptosis, and/or DNA repair) (reviewed in Friedberg et al., 2000; Hakem and Mak, 2001; Gartel and Tyner, 2002; Hollander and Fornace, 2002; Wang et al., 2004). XPC and p21 can modify the *Trp53*- and *Gadd45a*-null exencephaly phenotype, respectively, effectively adding to the genetic liability and reducing the threshold for exencephaly development. Our approach does have its limitations, since we have assumed that each pathway is linear without cross-talk. Therefore, although the approach may not directly implicate a specific pathway, it is possible to group candidate proteins based on their genetic liability to exencephaly in the double-null animals. Further biochemical analyses of these pathways during neural tube development will be required before definitive conclusions can be drawn.

Although many proteins are known to play critical roles, the detailed mechanisms by which NTDs form remain elusive. Genetic approaches using mouse models for NTDs

Table 1
Increased Spontaneous Exencephaly in *Gadd45a/Trp53*- and *Gadd45a/Cdkn1a*-null Mice

Genotype	No. of litters	No. of affected litters	No. of null embryos	No. (%) of embryos with exencephaly	No. (%) of embryos with spina bifida (SB) or curly tail (CT)
Gadd45a-null	17	6	119	10 (8.4)[a,b]	0[c]
Trp53-null	12[d]	3	33	3 (9.1)[a]	0
Cdkn1a-null	7	0	53	0	0
XPC-null	10	0	71	0	0
Gadd45a/Trp53-null	14[d]	11	47	16 (34.0)	1 CT (3.0%)
Gadd45a/Cdkn1a-null	12	12	105	32 (30.5)	3 SB (2.9%)
Gadd45a/XPC-null	9	1	68	1 (1.5)	0

[a]Significantly different from *Gadd45a/Trp53*-null using Fisher's exact test ($P < .05$).
[b]Significantly different from *Gadd45a/Cdkn1a*-null using Fisher's exact test ($P < .05$).
[c]Curly tail was observed on rare occasions but was not found in this dataset.
[d]Crosses of $Gadd45a^{+/-}/Trp53^{-/-}$, $Gadd45a^{-/-}/Trp53^{-/-}$, or $Gadd45a^{-/-}/Trp53^{+/-}$.

have provided a unique means of deciphering these pathways and have implicated apoptosis, genomic stability, cell-cycle control, and/or DNA repair as some of the pathways for normal neural tube development and closure. However, how these processes cooperate is generally not understood. Genetic approaches may help to elucidate more functional groups of proteins and, by assessing the association between these proteins, identify the mechanisms by which they monitor and/or regulate neural tube development.

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