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The molecular and cellular basis of variable craniofacial phenotypes and their genetic rescue in *Twisted gastrulation* mutant mice

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ABSTRACT

The severity of numerous developmental abnormalities can vary widely despite shared genetic causes. Mice deficient in *Twisted gastrulation* (*Twsg1*$^{-/-}$) display such phenotypic variation, developing a wide range of craniofacial malformations on an isogenic C57BL/6 strain background. To examine the molecular basis for this reduced penetrance and variable expressivity, we used exon microarrays to analyze gene expression in mandibular arches from several distinct, morphologically defined classes of *Twsg1*$^{-/-}$ and wild type (WT) embryos. Hierarchical clustering analysis of transcript levels identified numerous differentially expressed genes, clearly distinguishing severely affected and unaffected *Twsg1*$^{-/-}$ mutants from WT embryos. Several genes that play well-known roles in craniofacial development were upregulated in unaffected *Twsg1*$^{-/-}$ mutant embryos, suggesting that they may compensate for the loss of TWSG1. Imprinted genes were overrepresented among genes that were differentially expressed particularly between affected and unaffected mutants. The most severely affected embryos demonstrated increased p53 signaling and increased expression of its target, *Trp53inp1*. The frequency of craniofacial defects significantly decreased with a reduction of *p53* gene dosage from 44% in *Twsg1*$^{-/-}$*p53*$^{+/+}$ pups (N = 675) to 30% in *Twsg1*$^{-/-}$*p53*$^{+/-}$ (N = 47, p = 0.04) and 15% in *Twsg1*$^{-/-}$*p53*$^{-/-}$ littermates (N = 39, p = 0.001). In summary, these results demonstrate that phenotypic variability in *Twsg1*$^{-/-}$ mice is associated with differential expression of certain developmentally regulated genes, and that craniofacial defects can be partially rescued by reduced p53 levels. We postulate that variable responses to stress may contribute to variable craniofacial phenotypes by triggering differential expression of genes and variable cellular apoptosis.

Introduction

Phenotypic variation is a well-known phenomenon in a number of developmental abnormalities. For example, patients with Treacher-Collins syndrome or holoprosencephaly (HPE) have an extremely wide range of clinical manifestations (Hansen et al., 1996; Muenke and Cohen, 2000; Roessler et al., 1996). In HPE, the most severe defects, such as cyclopia, are usually incompatible with life, while others including hypotelorism or microphthalmia represent milder forms (Ming et al., 2002). However, the molecular basis for such reduced penetrance and variable severity, even in the context of the same mutations on the same genetic background, has remained unclear.

Differences in genetic background and environmental influences are thought to be two major drivers of transcriptional variation, leading to phenotypic variation or reduced penetrance in both humans and mice (Butchbach et al., 2009; Champy et al., 2008). The contribution of epigenetic phenomena, including DNA methylation and histone modification, has also been increasingly recognized to be an important mechanism underlying variability in genetic expressivity, particularly in the case of imprinted genes (Dindot et al., 2009;

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Peaston and Whitelaw, 2006). For example, craniofacial manifestations of the Silver-Russell syndrome correlate with the degree of methylation at the H19-(Insulin-like Growth Factor) IGF2 locus (Bruce et al., 2009). This underscores the importance of a better understanding of the basis of phenotypic variation of craniofacial malformations and identification of mammalian models that would facilitate such insights. An advantage in using inbred mouse models to study such phenotypic variation is that both strain differences and environmental differences can be controlled.

We previously reported that disruption of *Twisted gastrulation* (*Twsg1*), a modulator of BMP signaling, in mice leads to a spectrum of craniofacial defects on an isogenic, inbred C57BL/6 background (MacKenzie et al., 2009; Petryk et al., 2004). Within a single litter, some mice are born with normal craniofacial structures; some have mild defects, such as micrognathia and microphthalmia; and still others have profound craniofacial abnormalities including anterior truncation, cyclopia, agnathia, and HPE. While some of this variation could be attributed to a variable extent of apoptosis and thus variable loss of first branchial arch 1 (BA1) derivatives (MacKenzie et al., 2009), the underlying molecular mechanisms are not entirely understood. Manifestation of craniofacial defects is highly strain background-dependent and appears in about 40% of $Twsg1^{-/-}$ mice on the C57BL/6 background. In early generations after derivation of the mutant allele on the C57BL/6 background, some as yet undiscovered modifier gene/s could partially account for the observed variation. However, this variation has persisted despite serial backcrossing of the mutant alleles onto the isogenic strain background. The ongoing occurrence of extreme differences in phenotypes, even within the same litter, raises the question of how phenotypic variation might occur despite virtually no differences in inherited genes. The *Twsg1* mutant mouse model provides an excellent, well-controlled experimental system in which to study the molecular mechanisms underlying the resulting phenotypic variation and reduced penetrance.

In this study, we have analyzed transcriptional changes that are associated with development of distinct classes of craniofacial phenotypes in *Twsg1* mutant mice. We anticipated that these differentially expressed transcripts would include previously unknown effectors of the mutant phenotype as well as genes that may compensate for the loss of TWSG1 and account for incomplete penetrance of *Twsg1* mutation. In particular, we were interested in identifying transcriptional differences between unaffected and affected mutants that would shed light on potential mechanisms that allow an organism to cope with a predisposition to disease.

Material and methods

Mice and tissue collection

Generation and genotyping of mice deficient for TWSG1 (Petryk et al., 2004), p53 (Donehower et al., 1992) as well as transgenic *Sox10Cre* mice that express *Cre* recombinase in neural crest cells (NCC) (Matsuoka et al., 2005) have been previously reported. To generate mice with NCC-specific deletion of *Twsg1* ($Twsg1^{flox/flox}$; *Sox10Cre*), mice carrying a conditional allele of *Twsg1* ($Twsg1^{flox/flox}$), with exon 4 (coding exon 3) flanked by *loxP* sites, were mated to *Sox10-Cre* mice to generate $Twsg1^{flox/WT}$; *Sox10Cre*, which were then mated to $Twsg1^{flox/flox}$ mice. All strains were on C57BL/6 background. Presence of a spermatic plug was counted as day 0.5 post conception (E0.5). $Twsg1^{-/-}$ embryos were classified as previously published as class A (phenotypically the same as wild type), class B (moderately affected), and class C (severely affected) (MacKenzie et al., 2009). For microarray analysis, mandibular prominences of BA1s from mutants of various phenotypic classes and WT embryos at E10.5 were dissected by cutting the BA1 at the junction between the maxillary and mandibular components using alkali etched tungsten needles. BA1s were flash-frozen in liquid nitrogen and stored at −80 °C. Both maxillary and mandibular prominences were dissected at E11.5 to confirm deletion of exon 4 of *Twsg1* in NCC-derived tissues. This embryonic stage was chosen to allow the analysis of individual samples in case of variable efficiency of *Cre*-mediated recombination. Use and care of the mice in this study was approved by the University of Minnesota Institutional Animal Care and Use Committee.

RNA isolation

Frozen tissue samples were thawed in Trizol reagent (Invitrogen, Carlsbad, CA) and pooled according to the phenotypic class. RNA was purified using RNeasy micro kit spin columns (Qiagen, Valencia, CA). Five biological replicates of each mutant class and of WT (each representing a pool of 4–7 arches) were analyzed to achieve adequate statistical power for this microarray analysis (Pavlidis et al., 2003). Pooling was necessary to allow collection of sufficient RNA for microarray analysis (Bobola et al., 2003; Feng et al., 2009).

Microarray analysis

Microarray analysis was performed at the Kimmel Cancer Center Cancer Genomics Core Facility, Thomas Jefferson University (Philadelphia, PA). Total RNA (50 ng) was used to prepare amplified cDNA using the WT-Ovation Pico RNA amplification system (NuGen Technologies, Inc., San Carlos, CA). Sense transcript cDNA (ST-cDNA) was generated from 3 μg of amplified cDNA. Finally, 5.0 μg ST-cDNAs were labeled using FL-Ovation cDNA biotin module v2 (NuGen Technologies, Inc.) (Linton et al., 2009). Affymetrix GeneChip mouse exon 1.0 ST arrays (Affymetrix, Santa Clara, CA) were used following manufacturer's recommendations. Samples were hybridized overnight, scanned and processed using Command Console Software. Background correction and normalization were done using Robust Multichip Average (RMA) with Genespring v10.0 software (Agilent, Palo Alto, CA, USA).

Bioinformatics analysis

mRNA expression profile data were condensed using RMA to generate raw expression values log base 2. Two group T-tests were used to assess significance in pairwise comparisons between groups with correction for a false discovery rate (FDR) of 10% or less (Benjamini and Hochberg, 1995). For inclusion in WT vs. C and A vs. C analyses, genes were also required to have an absolute value of fold change ≥ 1.5. Heatmaps were generated using Cluster3.0 and JavaTreeview. Expression profiles were clustered in both supervised and unsupervised methods to identify molecular patterns present in the data. Ingenuity Pathways Analysis (IPA; Ingenuity® Systems, www.ingenuity.com) was used to identify biological functions regulated by differentially expressed genes.

Quantitative PCR

Reverse transcription was carried out with the Thermoscript RT kit (Invitrogen) priming with random hexamers, followed by quantitative PCR (Q-PCR, MX3000p, Agilent, LaJolla, CA) using expression assays from Applied Biosystems (Foster City, CA) for *Bambi* (Mm03024088_g1), *Bmp4* (Mm00432087_m1), *Bmpr1b* (Mm03023971_m1), *Cyp26a1* (Mm00514486_m1), *Dkk1* (Mm00438422_m1), *Gpr50* (Mm00439147_m1), *Peg3* (Mm00493299_s1), *Plagl1* (Mm00494250_m1), *Satb2* (Mm00507337_m) or using published primer sequences with SYBR green RT^2 master mix (SABiosciences, Valencia, CA) for *Dlk1*, *Igf2* (Varrault et al., 2006), and *Msx2* (Berdal et al., 2009). To detect *Twsg1*, the following primers were used (forward: 5′-CTGAACTGGAACATCGTCTC-3′, reverse: 5′-GCAGTCATCAAAG-TAAACCAC-3′). *Trp53inp1*was detected with primers from the MGH