22q11 Gene dosage establishes an adaptive range for sonic hedgehog and retinoic acid signaling during early development

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Received July 3, 2012; Revised September 4, 2012; Accepted October 8, 2012

We asked whether key morphogenetic signaling pathways interact with 22q11 gene dosage to modulate the severity of cranial or cardiac anomalies in DiGeorge/22q1 deletion syndrome (22q11DS). Sonic hedgehog (Shh) and retinoic acid (RA) signaling is altered in the brain and heart—clinically significant 22q11DS phenotypic sites—in LgDel mouse embryos, an established 22q11DS model. LgDel embryos treated with cyclopa mine, an Shh inhibitor, or carrying mutations in Gli3Xtj, an Shh-signaling effector, have morphogenetic anomalies that are either not seen, or seen at significantly lower frequencies in control or single-mutant embryos. Similarly, RA exposure or genetic loss of RA function via heterozygous mutation of the RA synthet ic enzyme Raldh2 induces novel cranial anomalies and enhances cardiovascular phenotypes in LgDel but not other genotypes. These changes are not seen in heterozygous Tbx1 mutant embryos—a 22q11 gene thought to explain much of 22q11DS pathogenesis—in which Shh or RA signaling has been similarly modified. Our results suggest that full dosage of 22q11 genes beyond Tbx1 establish an adaptive range for morphogenetic signaling via Shh and RA. When this adaptive range is constricted by diminished dosage of 22q11 genes, embryos are sensitized to otherwise benign changes in Shh and RA signaling. Such sensitization, in the face of environmental or genetic factors that modify Shh or RA signaling, may explain variability in 22q11DS morphogenetic phenotypes.

INTRODUCTION

DiGeorge or 22q11 deletion syndrome (22q11DS) is the consequence of a hemizygous loss of a ‘critical’ (1.5 Mb) or larger ‘typical’ (3 Mb) region of human Chr.22 (1). Clinically significant 22q11DS phenotypes vary in penetrance and severity. They include modest to life-threatening cardiovascular malformations (2,3), mild-to-severe craniofacial and limb anomalies (2), parathyroid and thymic hypoplasia (4) and increased susceptibility to a range of behavioral and psychiatric disorders from autism and intellectual disability to schizophrenia (5). Despite compelling clinical data on varying frequency and severity of 22q11DS phenotypes, there is little mechanistic insight into why shared genomic lesions result in variable outcomes (6,7). Accordingly, we asked whether key morphogenetic signaling pathways, whose activity and influence may vary at 22q11DS phenotypic sites, interact with diminished 22q11 gene dosage to modulate the severity of cranial or cardiac anomalies.

Initial differentiation at all 22q11DS phenotypic sites, including the brain and heart, depends upon morphogenetic interactions between mesenchymal and epithelial tissues mediated by diffusible signals including sonic hedgehog (Shh), retinoic acid (RA), fibroblast growth factors (Fgfs)
and bone morphogenetic proteins (Bmps) (8). Coincident expression of 22q11 genes with these signals in the brain, heart, face and limbs (9–11) suggests that Shh, RA, Fgfs, and Bmps are potential modifiers of 22q11DS phenotypes. Indeed, some phenotypes in mutant mice with genetic disruption of RA (Raldh2) and Fgf8 signaling have been identified as 22q11DS ‘phenocopies’ (12,13), whereas the consequences of Shh and Bmp signaling anomalies are more narrowly interpreted as ‘parallel’ to 22q11DS (14,15). It is unclear, however, whether such similarities reflect linear effects on common downstream targets consistent with ‘phenocopy’, or more complex interactions between local signals and 22q11 gene dosage. Shh and RA apparently regulate expression of at least one 22q11 gene, Tbx1, when deleted in combination with Crlk (outside the critical region, but within the typical region; 1), modulates RA and Fgf8 signaling (18), while total loss of Tbx1 function down-regulates Bmp4 (19). Moreover, mutations in RA- as well as Fgf8- and Bmp-associated signaling genes modulate Tbx1 mutant phenotypes (20–23). Tbx1 has been robustly associated with several 22q11DS anomalies including aortic arch, thymus and palatal anomalies (24–26). Nevertheless, the role of Tbx1 in the full 22q11DS spectrum remains uncertain (9,27–29). Thus, we compared the consequences of broader 22q11 deletion versus heterozygous Tbx1 mutation for altering key morphogenetic signaling pathways and essential 22q11DS phenotypes.

We found that interactions between Shh or RA signaling and 22q11 gene dosage enhance 22q11DS-related phenotypes in the LgDel mouse 22q11DS model but not Tbx1 mutant mice. Alterations of Shh or RA signaling that are otherwise benign yield more frequent and severe cranial or cardiovascular anomalies in LgDel mice. Our results do not support simple, linear relationships between morphogenetic signals and 22q11 genes. Instead, 22q11 gene dosage, Shh and RA likely participate in broader homeostatic networks that modulate a dynamic range of signaling for adaptive morphogenesis in the brain, heart and other 22q11DS phenotypic sites. Disruption of these networks by environmental factors or genetic polymorphisms may be an essential contributor to phenotypic variability in 22q11DS.

RESULTS

Diminished 22q11 dosage disrupts inductive signaling pathway gene expression

22q11 gene dosage might influence Shh, RA, Fgf or Bmp signaling at 22q11DS phenotypic sites during initial morphogenesis. To assess this potential relationship, we used quantitative PCR to compare expression levels of a subset of Shh, Fgf or Bmp ligands, RA synthetic enzymes, as well as receptors and co-factors for each signal—all with selective expression at 22q11DS phenotypic sites (8)—in LgDel, Tbx1+/- and wild-type (WT) littermate embryos for each genotype at embryonic day (E) 10.5, a critical stage for morphogenetic interactions.

Nine of 10 representative Shh-signaling genes are diminished by 18–25% in the E10.5 LgDel embryo: Shh itself, the Ptc1 co-receptor, Smo, an intracellular mediator, Gli1, Gli2 and Gli3 transcriptional activator/repressors, and co-factors Hhip, Sufu and Boc (n = 7 LgDel, 7 WT; 8 genes P <= 0.05; 2 trends, P <= 0.06; Fig. 1). Five of nine representative RA-signaling genes are diminished by 28–32%: synthetic enzymes Raldh2 and Raldh3, RA receptors Rara and Rarb and the RA catabolic enzyme Cyp26a1 (n = 7 LgDel, 7 WT; 4 genes P <= 0.05; 1 trend P <= 0.06). Two of eight Fgf-related genes decline: the Fgf receptor Fgfr1 and the Fgf target Mkp3 (10–18%; n = 7 LgDel, 7 WT; 1 gene, P <= 0.05; 1 trend P <= 0.06). Finally, six of 13 Bmp-related genes are diminished by 21–39%: four ligands (Bmp2, 4, 5 and 7), the activin receptor 1 Acvr1 (a non-selective Tgfβ receptor), the Bmp receptor Bmpr1 and the non-selective transcriptional mediator Smad4 (n = 7 LgDel, 7 WT; 6 genes P <= 0.05).
Diminished 22q11 dosage alters Shh and RA signaling

The decline in the expression of signaling pathway intermediates does not necessarily mean that signaling via these pathways is altered. Thus, we measured local, independent ‘output’ of Shh and RA signaling—two pathways highly related to 22q11 genes based on previous observations (16–18)—in the head/forebrain and heart in LgDel as well as Tbx1+/- embryos. We focused on signaling levels at these sites, and excluded regions like the spinal cord and limb bud where Shh and RA signaling is robust, but 22q11DS phenotypes are less well defined. Thus, we can determine whether there are regional differences in the consequences of diminished 22q11 gene expression for morphogenetic signaling at distinct locations known to be compromised in 22q11DS.

For Shh, we crossed a ‘knock-in’ Ptc2:β-galactosidase (βgal) reporter (34) into LgDel and Tbx1+/- to visualize and quantify local signaling independent of mRNA levels, which may vary based upon stability. There are neither visible changes in Ptc2:βgal patterns nor quantifiable changes in activity (measured by soluble βgal in dissected samples) in the LgDel head/forebrain (Fig. 2A and B, top), and there are no changes in Ptc2, Shh or Gli1 mRNA levels (n = 8 LgDel, 9 WT; Fig. 2C, top). In contrast, Ptc2:βgal expression expands in the LgDel heart/aortic arches (Fig. 2A and B, bottom), and we detect a 25% increase in soluble βgal activity in isolated samples of the cardiac region (n = 5 LgDel, 5 WT; P ≤ 0.05, Fig. 2B). We found a parallel increase of Ptc2 mRNA (45%; n = 7 LgDel, 9 WT; P ≤ 0.01), and Shh itself is increased by 57% (n = 7 LgDel, 7 WT; P ≤ 0.019; Fig. 2C). These changes are not seen in Tbx1+/- embryos for Ptc2:βgal activity (n = 5 Tbx1+/-; 4 WT; Fig. 2D and E) or Ptc2, Shh or additional Shh-related signaling genes (n = 7 Tbx1+/-; 9 WT; Fig. 3D and data not shown).

To measure RA signaling, we bred an RA-indicator transgene (DR5-RARE; 35), which is quantitatively sensitive to altered RA levels (Supplementary Material, Fig. S2), into LgDel and Tbx1+/- embryos. In LgDel embryos, DR5-RARE-dependent RA signaling appears modestly diminished, based upon altered intensity and extent of βgal labeling, in the head/forebrain and heart/aortic arches (Fig. 3A). In parallel, RA signaling levels (soluble βgal activity in dissected samples) decrease modestly but significantly in the head/forebrain (12%; n = 6 LgDel; 6 WT; P ≤ 0.05) and heart (15%; P ≤ 0.05, Fig. 3B). Expression levels of RA synthetic

Figure 2. Shh signaling is increased in the developing heart in LgDel, but not Tbx1+/- E10.5 embryos. Embryos in (A) and (D) carry a β-galactosidase (βgal) reporter under the control of the endogenous Ptc2 promoter. (A) Comparison of Ptc2:βgal labeling in the head/forebrain (fb, including the eye, e; top panels; dotted line indicates site of dissection) and heart/aortic arches (H/AA; dotted lines indicated site of dissection) of E10.5 WT (left) and LgDel embryos (right). βgal labeling in the nascent heart and aortic arches (arrowheads) is less extensive in WT than LgDel embryos (8 LgDel, 10 WT embryos analyzed). There is no apparent change in the forebrain/head. Dotted lines in left hand panels indicate regions dissected for quantitative measurements. (B) Increased Ptc2 promoter activation in the heart and aortic arches of the LgDel embryos, without change in the head/forebrain, assessed by soluble βgal levels (ONPG assay, see Materials and Methods). (C) Increased Ptc2 and Shh mRNA levels in the LgDel heart and aortic arches (HAA; solid yellow bars; LgDel values plotted as fold change from WT values = 1), but not in the head/forebrain. (D) No visible difference in cranial or cardiac βgal labeling in WT and Tbx1+/- E10.5 embryos. (E) Equivalent levels of βgal activity in the head/forebrain and heart/aortic arches of WT and Tbx1+/- embryos. (F) No significant change in Shh or Ptc2 expression in either head or heart of Tbx1+/- embryos.
enzymes and receptors parallel these changes (Fig. 3C); Ranbp1 declines significantly in the heart (37%; n = 7 LgDel, 7 WT; P ≤ 0.04) and RanB expression declines significantly in both the head/forebrain and heart (32 and 56%, respectively; n = 7 LgDel, 7 WT; P ≤ 0.05). In contrast, in Tbx1+/- embryos, DR5-RARE:βgal activity does not change significantly at either site (n = 6 Tbx1+/-; 4 WT; Fig. 3D and E). There are no significant changes in the expression levels of Ranbp1 and RanB in the head/forebrain of Tbx1+/- embryos; however, Ranhd2 declines significantly in the heart (22%, n = 7 Tbx1+/-, 9 WT; P ≤ 0.003), in parallel with that in the LgDel.

Together, these results establish modest but statistically significant changes as well as local variation of Shh and RA signaling at key 22q11DS phenotypic sites—the forebrain/head and heart in LgDel, but not Tbx1+/-, embryos. In one case—Shh signaling in the heart—local changes are distinct from those detected in whole embryos (Fig. 1). In contrast, changes of RA signaling and related gene expression in the forebrain/head and heart parallel those measured in the whole embryo.

**Ssh, RA, Fgf and Bmp signaling influence 22q11 gene expression**

Morphogenetic signaling is interactive and homeostatic—signals influence and are modulated by multiple target genes—perhaps to adjust for individual genetic and environmental variation (36,37) including subtle changes in signaling like those we found in LgDel embryos. Given the coincident expression of multiple 22q11 genes and morphogenetic signals, it is possible that Shh, RA, Fgfs or Bmps regulate expression of 22q11 genes beyond Tbx1 (16,17). Thus, we asked if morphogenetic signals regulate 22q11 gene expression during early embryogenesis.

We did an initial comparison of mRNA levels of 22 22q11 genes selectively expressed at 22q11DS phenotypic sites (11) in whole E10.5 embryos carrying constitutive loss- and gain-of-function mutations for morphogenetic signals, or after acute exposure to function blocking agents (a total of 11 genetic or pharmacological manipulations for which we measured expression of 22 22q11 genes plus several controls; n = 3 for each mutation or manipulation; Supplementary Material, Fig. S3). This initial analysis identified a subset of genes for each signaling pathway that are consistently and substantially altered by loss or gain of function. From this subset, we selected a small group of 22q11 genes for in situ hybridization (ISH) combined with further qPCR analysis to determine whether local patterns of 22q11 gene expression change in response to pharmacologically increased or decreased morphogenetic signaling in WT embryos. For each of four 22q11 genes (Fig. 4), we saw local expression level changes at 22q11DS phenotypic sites (ISH performed on sets of WT treated and untreated embryos in the same vials; n ≥ 4 WT and treated embryos for each gene) whose direction and magnitude is consistent with qPCR measurements. Expression patterns of these genes were not dramatically expanded or contracted and there were no novel or ectopic expression domains. For some genes, however, expression level changes were not uniform at all 22q11DS phenotypic sites—for example, there is increased Ranbp1 labeling in the frontonasal process and limb, but not branchial arches or heart following cyclopamine exposure (Fig. 4, top). Evaluation following FgfR inhibition is complicated by altered growth at 22q11DS phenotypic sites (Fig. 4, middle). Together, these results show that some regulation of 22q11 genes by Shh, RA, Fgf and Bmp is possible. This regulation, however, likely reflects local modulation of expression levels—or overall growth—at 22q11DS phenotypic sites rather than substantial changes in patterning of the embryo.

**22q11 gene dosage and Shh signaling interact during morphogenesis**

Our data suggest that interactions between 22q11 gene dosage and local signals influence morphogenesis at 22q11DS phenotypic sites. Environmental or genetic changes, beyond 22q11 deletion but acting on shared targets, could lead to enhanced, diminished or novel phenotypes. Alternately, additional disruption of 22q11 gene expression by dis-regulated signaling might yield loss of function, or dosage rescue of one or more of 22q11 genes with parallel key 22q11DS phenotypic

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**Figure 3.** Diminished RA signaling in the head/forebrain and heart/aortic arches in LgDel, but not Tbx1+/- E10.5 embryos. Embryos in (A) and (D) carry DR5-RARE:βgal RA reporter transgene. (A) βgal labeling in the forebrain (fb) and eye (e; top panels) and the heart/aortic arches (H/AA; dotted lines) in E10.5 WT and LgDel embryos. βgal labeling is diminished in the forebrain/head (compare arrowheads, top right) and aortic arches (compare arrowheads, bottom right) of LgDel versus WT (9 WT and 6 LgDel embryos analyzed). (B) Diminished βgal activity in dissected LgDel head and heart based upon soluble βgal assays (ONPG). (C) RarB expression declines significantly in the forebrain/head in LgDel embryos, while both Ralhd2 and RarB expression decline in the heart (solid purple bars). (D) No visible differences in cranial or cardiac βgal labeling in Tbx1+/- versus WT embryos. (E) βgal activity (ONPG assay) is statistically equivalent in Tbx1+/- and WT littermates (F) Expression levels of Ralhd2 and RarB in the head and heart are statistically indistinguishable in Tbx1+/- and WT littermates.
changes. Thus, we altered Shh signaling levels in LgDel and WT littermates between E8.5 and E10.5 with sub-teratogenic doses of cyclopamine (38) or Shh and Gli3 mutations, and assessed consequences for morphogenesis and gene expression.

Cyclopamine-exposed LgDel embryos are far more compromised than WT littermates (Fig. 5A; n = 9 LgDel, 8 WT, two litters). Most cyclopamine-treated LgDel embryos (77%) fail to develop limb buds, aortic or branchial arches, olfactory placodes, eyes and forebrains, whereas cyclopamine-treated WT embryos, even though some are dysmorphic, develop these structures. In parallel, there is an equivalent decline (80%) of Shh-regulated Ptc2 in LgDel and WT embryos (Fig. 5B; n = 3 LgDel, 3 LgDel + cyclo., 3 WT, 3 WT + cyclo.).
Additional Shh signaling genes, however, are significantly more diminished in cyclopamine-exposed LgDel embryos, including Ptc1 (93% LgDel; 68% WT; \( P \leq 0.01 \)), Smo (96% LgDel; 88% WT; \( P \leq 0.02 \)) and Gli1 (90% LgDel; 75% WT; \( P \leq 0.003 \)). In contrast, 22q11 gene expression—except for Zdhhc8 (for which homozygous deletion does not result in obvious morphogenetic change; 39)—is not diminished beyond 50% in cyclopamine treated LgDel embryos (Fig. 5C; \( n = 3 \) LgDel + cyclo, 3 LgDel; \( P \leq 0.0002 \) for Zdhhc8), including 22q11 genes that change in response to cyclopamine in WT embryos (Fig. 4 and Supplementary Material, Fig. S3). Finally, cyclopamine-treated Thx1 \(^{+/−} \) embryos do not differ morphologically from WT littermates (\( n = 7 \) Thx1 \(^{+/−} \); \( n = 9 \) WT embryos from two litters; Fig. 5D). Apparently, diminished 22q11 gene dosage—beyond Thx1—sensitizes mid-gestation embryos to deleterious effects of substantially reduced Shh activity. Cyclopamine-induced dysmorphology in WT embryos from Thx1 litters is less noticeable than in WT embryos from LgDel litters, independent of genetic background (Fig. 5D). This may reflect systemic maternal responses due to the resorption of cyclopamine-exposed LgDel embryos that also compromise WT littermates (40, 41).

We confirmed this apparent interaction between Shh and 22q11 dosage genetically using Shh \(^{+/−} \) (42), Gli3 \(^{+/−} \) and Gli3 \(^{−/+\times X0} \) (43) mutants in combination with LgDel. For rigorous assessment of phenotypic modulation, we quantified a specific 22q11DS morphogenetic phenotype seen clearly in LgDel embryos—4th pharyngeal arch artery hypoplasia (PA4; Fig. 6A). In Shh \(^{+/−} \), expression of Smo, Ptc1 and Ptc2, all Shh-regulated (44), are not altered (\( P \geq 0.4 \), \( n = 5 \)), even though Shh declines by 40% (\( P \leq 0.007 \), \( n = 6 \) Shh \(^{+/−} \); 4 WT), and Gli3 increases by 16% (\( P \leq 0.03 \); Fig. 6B). We saw no gross phenotypic changes in LgDel:Shh \(^{+/−} \) embryos (data not shown). Forty-four percent (4/9) of Shh \(^{+/−} \)-LgDel embryos have a hypoplastic or absent PA4, statistically indistinguishable from the 57% PA4 frequency in LgDel littermates (\( P \leq 0.2 \); Table 1). We selected Gli3 as a likely Shh gain-of-function mutation based upon its established role in Shh-mediated repression (45). Surprisingly, Shh itself as well as two positive regulators of Shh signaling are reduced rather than increased—the anticipated consequence of release of repression—in Gli3 \(^{+/−} \) and Gli3 \(^{−/+\times X0} \) embryos (Fig. 6C). In Gli3 \(^{+/−} \) embryos, Gli1 was reduced to 61% of WT (\( P \leq 0.03 \), \( n = 10 \) Gli3 \(^{+/−} \); 8 WT). In Gli3 \(^{−/+\times X0} \) embryos, Gli1, Smo and Shh were reduced to 29, 53 and 60% of WT (\( P \leq 0.02 \), \( n = 5 \) Gli3 \(^{−/+\times X0} \); 8 WT), respectively. Together these data suggest that Gli3 mutation results in diminished, rather than enhanced Shh signaling. One hundred percent of Gli3 \(^{−/+\times X0} \)-LgDel (\( n = 8/8 \)) and Gli3 \(^{−/+\times X0} \)-LgDel (\( n = 6/6 \)) embryos have significant PA4 disruptions seen at significantly lower frequency or not at all in WT, LgDel or Gli3 mutants (Fig. 6D; Gli3 \(^{−/+\times X0} \)-LgDel \( P \leq 0.005 \); Gli3 \(^{−/+\times X0} \)-LgDel \( P \leq 0.01 \); Table 1). Thus, in LgDel embryos, diminished Shh in Shh \(^{+/−} \) mutants is insufficient to modify 22q11DS phenotypes, whereas decreased Shh signaling in cyclopamine treated LgDel as well as Gli3 \(^{−/+\times X0} \)-LgDel embryos reaches a threshold for phenotypic change not seen in WT littermates, single mutants or Thx1 \(^{+/−} \) embryos.

22q11 gene dosage and RA signaling interact during morphogenesis

If a proposed ‘phenocopy’ of 22q11DS due to RA loss-of-function (13) reflects linear relationships between RA signaling and 22q11 gene dosage, enhanced signaling should
result in rescue, and decreased signaling should have little influence on 22q11DS phenotypes. This would be consistent with diminished levels of RA signaling we have found in LgDel embryos. If, however, the relationship between 22q11 and RA signaling reflects more complex homeostatic interactions with 22q11 gene dosage, either raising or lowering RA signaling could lead to significantly enhanced or novel phenotypes.

Modest, sustained doses of \textit{all trans} RA delivered via maternal circulation between E8.5 and E10.5, similar to those reported to rescue RA-related mutant phenotypes [46], substantially increase RA signaling levels. Based on DR5-RARE quantification, 48 h RA exposure increases RA signaling in the head/forebrain by 73\% for WT (\(n = 4; P \leq 0.02\)) and 50\% for LgDel embryos (\(n = 4; P \leq 0.02\)). In the heart, there is a divergent response: LgDel embryos have a significant increase (38\%, \(n = 4, P \leq 0.03\)); WT embryos do not (18\%, \(n = 4, P \geq 0.14\)). Thus, diminished 22q11 gene dosage differentially modulates cranial versus cardiac RA signaling. These changes are accompanied by disruption of cranial and cardiovascular morphogenesis in RA-exposed LgDel embryos not seen in RA-exposed WT littersates or untreated LgDel embryos (Fig. 7A). RA-treated LgDel embryos have significantly increased neural tube closure defects—a phenotype not routinely associated with 22q11DS [but see Nickel and Magenis (47)] 11/15 LgDel + RA versus 0/39 WT + RA and 0/33 LgDel untreated are exencephalic (\(P \leq 0.0001\); Table 2). Cardiac phenotypes are also modified. PAA dysmorphology is substantially enhanced in RA-treated LgDel versus WT littersates or untreated LgDel embryos (Fig. 7B; Table 3). PAA4 defects reach 100\%, and PAA4 is frequently absent (5/8 in LgDel + RA; 0/8 LgDel; \(P \leq 0.01\); Fig. 7B, Table 3). These novel anomalies are not seen at significantly higher levels in RA-treated Tbx1\(^{+/-}\) embryos (Tables 2 and 3). Thus, diminished 22q11 gene dosage beyond Tbx1 interacts with elevated RA signaling in the developing brain and heart to yield substantially altered phenotypes.

We next asked whether increased RA signaling in the LgDel differentially modifies RA-signaling gene expression. Expression of several genes that are either unchanged (Raldh1) or diminished in LgDel embryos (Raldh2, Rara, Rar\(a\),...
(Fig. 8A). In each of the three genotypes, levels are often +33 +33 0 LgDel + WT WT 39 39 0 LgDel + RA + 20% diminished RA signaling levels based on scoring for exencephaly at E10.5. Table 2. The consequences of pharmacological or genetic alteration of RA signaling for neural tube closure in LgDel embryos, based on scoring for exencephaly at E10.5.

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Genotypes and RA treatment groups are identified at left, and the frequency of exencephaly is recorded at right.

Table 3. The consequences of pharmacological or genetic alteration of RA signaling for PAA4 morphogenesis in LgDel embryos

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Genotypes and RA treatment groups are identified at left, and the frequency of PAA dysmorphology, scored as hypoplastic or absent, is listed in the middle and right hand columns.

Rarβ; Fig. 1) is further decreased by RA exposure (Fig. 7C; n = 3 LgDel + RA, 3 LgDel, 3 WT + RA, 3 WT; P ≤ 0.04 Raldh1, 0.02 Raldh2, 0.005 Rars, 0.03 Rarβ). Indeed, for two RA-regulated retinoid receptors, Rarα and Rarβ, RA has divergent effects in WT and LgDel embryos. In contrast, despite some indication that RA influences 22q11 gene expression (Fig. 4 and Supplementary Material, Fig. S3), there is no significant change for any 22q11 genes in RA-treated LgDel embryos beyond the 50% diminished expression seen in untreated LgDel embryos (Fig. 7D; n = 3 LgDel + RA, 3 Lgdel; see also Supplementary Material, Fig. S3). Therefore, diminished 22q11 gene dosage differentially alters the transcriptional response to elevated RA signaling without modifying 22q11 gene expression.

Finally, we asked whether LgDel embryos are also sensitized to a genetic mutation that modestly lowers RA signaling. E10.5 mouse embryos carrying a heterozygous Raldh2 mutation (48) have 20% diminished RA signaling levels based on quantitative assessment using the DR5-RARE reporter (P ≤ 0.002, n = 4), without obvious dysmorphology. This change has consequences for RA-regulated gene expression in Raldh2+/− as well as Raldh2+/−:LgDel embryos (Fig. 8A). In each of the three genotypes, levels are often significantly lower than WT (asterisks); however, there are no significant differences between Raldh2+/− and Raldh2+/−:LgDel embryos. Moreover, selected 22q11 genes (apparently RA-regulated; Fig. 4 and Supplementary Material, Fig. S3) do not decline beyond the 50% LgDel values in either additional genotype (Fig. 8A, right). To determine if a potentially compound decrease of RA signaling due to heterozygous Raldh2 mutation and diminished 22q11 dosage results in novel or enhanced 22q11DS related phenotypes, we assessed neural tube closure and PAA4 development in Raldh2+/−:LgDel compound mutants. Thirty-six percent (4/11) of Raldh2+/−:LgDel mutants exhibit exencephaly not observed in LgDel, Raldh2+/− or WT embryos (P ≤ 0.02; Fig. 8B and Table 3). One hundred percent of Raldh2+/−:LgDel embryos have PAA4 dysmorphogenesis, whereas littermate LgDel embryos have the expected 57% frequency (Table 3; P = 0.02). In compound mutants, PAA4 phenotypes (Fig. 8C) include hypoplasia (4/8; 50%) as well as absence (4/8; 50%). These phenotypes are seen at significantly higher frequencies in Raldh2+/−:LgDel embryos versus single or compound Raldh2+/−:Tbx1+/− mutant embryos (P ≤ 0.01; Table 3; Fig. 8D and E). The frequency of PAA4
hypoplasia (66%) is statistically indistinguishable in Tbx1+/− and Raldh2+/−:Tbx1+/− embryos (n = 6/9 Raldh2+/−: Tbx1+/−; 4/10 Tbx1+/−; P = 0.2). Thus, diminished 22q11 gene dosage sensitizes LgDel, but not Tbx1+/− embryos to both elevated and diminished RA signaling, resulting in novel or more severe 22q11DS-related cranial and cardiac phenotypes.

**DISCUSSION**

When 22q11 gene expression is diminished, signaling via Shh and RA is altered in the developing brain and heart. These changes may directly influence embryonic phenotypes associated with 22q11DS; however, they also enhance sensitivity of cranial and cardiac development to additional Shh or RA signaling variation. Alteration of RA and Shh signaling that is minimally disruptive in WT or Tbx1 mutant embryos causes more frequent, severe or novel phenotypes in LgDel embryos. Apparently, full dosage of multiple 22q11 genes establishes an optimal adaptive range for Shh and RA signaling in the developing head and heart. 22q11 deletion constrains this range and diminishes embryonic tolerance to signaling variability. Thus, the mutable frequency and severity of 22q11DS phenotypes—particularly forebrain and heart anomalies—likely reflects, in part, consequences of otherwise relatively benign environmental exposure or genetic variation that alters morphogenetic signaling beyond adaptive levels that are constricted by diminished 22q11 gene dosage.

**Quantitative alterations of morphogenetic signaling in 22q11DS**

Four essential morphogenetic signaling pathways—activated by Shh, RA, Fgps and Bmps—have been associated with 22q11DS pathogenesis for the last two decades (12,13,15,16,18,23,33,49,50). Some pathways have been implicated based upon apparent phenotypic similarities in mutant mice and 22q11DS (49,50), while altered expression patterns of selected target genes, especially in Tbx1 mutant mice, indicate contributions by others (16–19,33,51). We found that diminished 22q11 gene dosage, beyond Tbx1, regulates expression levels of Shh, RA and Bmp—and to a lesser extent Fgf—signaling genes. These pathways in turn regulate 22q11 gene expression levels. This apparently reciprocal regulation, however, is limited. Altered Shh or RA signaling in LgDel embryos does not further reduce 22q11 gene expression (with one exception) beyond 50%. Finally, the magnitude or direction of changes in signaling and signal-dependent 22q11 gene expression varies at 22q11DS phenotypic sites including the brain and heart. Such distinctions provide a new framework for considering complex and variable 22q11DS phenotypes. Local quantitative changes in signaling and 22q11 gene dosage during mid-gestation likely modulate morphogenetic interactions between mesenchymal (M) and epithelial (E) tissues at these sites. Phenotypes may vary due to altered M/E signaling that idiosyncratically further modifies morphogenetic signaling activity in the context of diminished 22q11 dosage.

**22q11 gene dosage and Shh signaling interact during morphogenesis**

We found that diminished 22q11 gene dosage—exclusive of Tbx1—disrupts Shh signaling levels in the heart, and sensitizes embryos to deleterious effects of altered Shh signaling. In the heart, epithelial Shh may maintain a minimal level of 22q11 gene expression in the mesenchyme and establish feedback that further regulates epithelial Shh signaling centers (52). Diminished dosage of 22q11 genes may disrupt this relationship leading to the local increase in cardiac Shh and Shh signaling we found. This may reflect altered inductive capacity of neural crest derived cardiac mesenchyme (12,14,46,53) with 22q11-related epithelial disruption of Shh leading to additional mesenchymal changes. Shh is required for cardiac neural crest survival (54) and maintenance of heart field progenitor proliferation (55). Thus, the local gain in Shh message and Shh signaling in the heart (or stability in the forebrain despite embryo-wide diminished Shh expression in the LgDel) may very or modify some consequences of decreased 22q11 gene dosage (16,56). Indeed, compensatory changes in Shh signaling may be an M/E-mediated local response to embryo-wide Shh decline in the context of diminished 22q11 gene dosage.

When Shh signaling is disrupted briefly but substantially—based upon decline in levels of Ptc1, Ptc2 and Smo—following cyclopamine exposure, non-axial morphogenesis fails in LgDel but not WT or Tbx1+/− embryos. Therefore, diminished 22q11 gene dosage beyond Tbx1 sensitizes some essential aspect of Shh-mediated M/E-dependent morphogenesis at 22q11DS phenotypic sites. Cell proliferation may be a key target, since both Shh signaling and several 22q11 genes enhance proliferation (9,57). The combined consequences of cyclopamine-altered Shh signaling and diminished 22q11 dosage, especially in the mesenchyme where Shh is mitogenic (58) and 22q11 gene expression is enhanced (11) may overwhelm morphogenesis. Shh signaling is apparently diminished by Gli3 mutation based on declines in the Gli1 transcriptional activator (Gli13+/Xtj and Gli13−/Xtj) as well as Smo and Shh itself (Gli3−/Xtj). Partial or complete loss of Gli3 activator or repressor function may disrupt a precarious equilibrium of already modified Shh signaling leading to more severe phenotypes. Apparently, 22q11 deletion compromises the normal balance of Shh signaling in the heart, and thus alters the threshold for cardiac morphogenetic modulation by additional genetic or environmental changes in Shh signaling.

**RA signaling pathways are disrupted by 22q11 deletion**

Appropriate 22q11 gene dosage—again exclusive of Tbx1—maintains the integrity of RA signaling in LgDel embryos. Decreased expression of two out of three major RA synthetic enzymes, Raldh2 and Raldh3, essential for brain and heart development (59) as well as two RA-regulated receptors, Rarβ (60) and Rara, in LgDel embryos, all expressed primarily in the mesenchyme at 22q11DS phenotypic sites (11,52,61), may modify mesenchymal RA production (52,62) thus disrupting RA signaling in adjacent epithelia in LgDel embryos. Such changes might compromise proliferation or differentiation in progenitors that rely upon RA signaling in the
brain (63) and cardiac outflow tract (64). Thus, deletion of multiple 22q11 genes critical for RA signaling, especially 22q11 genes expressed selectively or exclusively in cranial and pharyngeal arch mesenchyme (11 and unpublished data), may interfere with the ability of cranial or cardiac tissues to generate, transmit or metabolize RA resulting in locally decreased RA signaling and brain and heart dysmorphogenesis.

We found that a sub-teratogenic increase in RA signaling (~50%) or a modest decline (20%) due to heterozygous mutation of Raldh2 has surprisingly similar morphogenetic consequences in LgDel but not WT, Raldh2+/- or Tbx1+/- embryos. This accords with many observations that demonstrate similar effects of increased or diminished RA signaling on morphogenesis (65). In both LgDel + RA and LgDel:Raldh2+/- embryos, RA-regulated genes have distinct transcriptional responses when 22q11 deletion and altered RA signaling are combined. Nevertheless, in both instances, there is novel occurrence of exencephaly as well as increased frequency and severity of PAA dysmorphogenesis. This consistent outcome, despite divergent RA-mediated transcriptional regulation, demonstrates—contrary to earlier claims based on studies in Tbx1 mutant embryos (18,33,66)—that singular changes in RA-sensitive genes like Krox20, Rara or other RA cofactors, are unlikely to explain specific cranial or cardiovascular phenotypes as 22q11DS phenocopies.

Morphogenetic disruptions in LgDel:Raldh2+/- embryos cannot be explained by modulation of RA signaling levels or related RA signaling gene expression alone. Indeed, expression of key RA signaling genes in the LgDel:Raldh2+/- compound mutant embryos is essentially indistinguishable from that in Raldh2+/- embryos. It is likely that transcriptional disruptions in the LgDel:Raldh2+/- embryos engage multiple targets including additional signaling pathways and downstream effectors. Diminished 22q11 gene expression alters key Shh and BMP signaling genes in parallel with changes in RA signaling. These pathways, whose activity is known to be modified in the forebrain or heart of Raldh2 mutant mice (67,68), and many others, might be more significantly compromised by simultaneous 22q11 gene deletion and heterozygous Raldh2 mutation than either alone. A broader analysis of transcriptome changes in single and compound mutants may identify such synergistic, quantitative disruptions of homeostatic regulation of networks beyond those directly engaged in RA signaling. Thus, additive disruptions, beginning with a single signaling pathway and expanding to include multiple gene networks, may contribute to phenotypic variation seen in 22q11DS.

**22q11 gene dosage establishes a dynamic range for morphogenetic interactions**

Our data show that full 22q11 gene dosage—exclusive of Tbx1—maintains an adaptive range for morphogenetic signaling in the heart and brain (Fig. 9). Accordingly, when 22q11 gene dosage is diminished in 22q11DS, or elevated, as in instances of 22q11 duplication (69,70), M/E interactions that drive heart and brain morphogenesis no longer accommodate modest to substantial changes in Shh and RA signaling that otherwise do not result in phenotypes. A broad range of altered Shh signaling, from 20 to 80% based on measurement of Ptc1, Ptc2, Gli1 or Smo (Fig. 9) is tolerated fairly well by WT, Shh+/−, Gli3+/− or Gli3−/− but not LgDel embryos. Similarly, a 50% increase or 20% decline defines a range of RA signaling that can be accommodated in the head and heart of WT and Tbx1+/-, but not LgDel embryos (Fig. 9). Thus, otherwise benign changes of Shh or RA signaling may explain a substantial amount of 22q11DS phenotypic variation (2). Such changes may arise from fairly common environmental and genetic disruptions (71,72), and these aberrations when combined with diminished 22q11 gene dosage likely modulate 22q11DS phenotypic severity. Diminished dosage of Tbx1 alone does not constrict the adaptive range for Shh and RA signaling; instead, diminished dosage of additional 22q11 genes—the fundamental change in 22q11DS patients—is necessary. Accordingly, deletion of 22q11 genes beyond Tbx1 likely interacts with otherwise benign alterations in morphogenetic signaling to enhance clinically significant phenotypes in individuals with 22q11DS.

**MATERIALS AND METHODS**

**Mice**

The University of North Carolina at Chapel Hill (UNC-CH) Division of Laboratory Animal Medicine or The George...
Quantitative PCR (qPCR)

E10.5 embryos (or dissected embryonic regions as described) were harvested, dissected and homogenized in TRIzol (Invitrogen). Total RNA was isolated, and cDNA synthesized and qPCR performed as described previously (10). Primers for qPCR are listed in Supplementary Material, Table S1. Expression of each transcript is displayed as the fraction of the qPCR are listed in Supplementary Material, Table S1. Expression of each transcript is displayed as the fraction of the expression in the WT and untreated cohort.

Statistical analysis

Mean expression values between genotypes or treatments were compared using Student’s unpaired t-test. Fisher’s exact tests, which might bias the work reported in the results, figures, and tables, or modify any interpretations, implications or opinions stated in the Results and Discussion. There have been no influences

Immunohistochemistry and ISH

Embryos were fixed overnight, and ISH and imaging was performed as described previously (9). For whole mount immunostaining, embryos were dehydrated in methanol, stored at -80°C and freeze-thawed (5 x). Specimens were rehydrated (PBS) and then incubated overnight at 4°C in 5% DMSO, 0.2% Triton X-100 and 5% normal goat serum in PBS. Following primary antibody incubation overnight at 4°C (Rat anti-CD31/PECAM; BD-Pharminigen), specimens were rinsed and labeled with Alexa-Fluor 488 or 546 conjugated anti-rat secondary antibodies overnight (4°C C). Finally, embryos were rinsed and dehydrated in MeOH, cleared with 2:1 benzyl alcohol:benzyl benzoate and imaged on a Zeiss LSM 510 confocal microscope. 2D projections of Z-Stacks were created using Zeiss LSM image processing software.

β-galactosidase staining and enzymatic activity quantification

Embryos were harvested and dissected in PBS and either fixed in 0.1% glutaraldehyde for whole mount staining or lysed in 2 × ONPG (ortho-Nitrophenyl-β-galactoside) lysis buffer (Promega) for soluble βgal assays as described previously (62). βGal activity was detected as a function of ONPG enzymatic hydrolysis analyzed at 420 nm on an ELX808 ultra microplate reader (Bio-Tek Instruments).

Pharmacological treatments

To evaluate regulation of 22q11 genes in WT embryos, pregnant WT dams (CD-1 strain) were injected with 10 mg/kg RA (Sigma), 100 mg/kg DEAB (Sigma), 80 mg/kg cyclopamine (LC Laboratories), 50 mg/kg PD173074 (Sigma) or 10 mg/ kg dorsomorphin (Sigma) at E9.5 and embryos harvested 24 h later. To evaluate consequences of disrupted Shh or RA signaling for morphogenesis, pregnant LgDel dams were injected with RA (10 mg/kg on E8.5 and 20 mg/kg on E9.5) or cyclopamine (80 mg/kg) twice (E8.5 and E9.5) over 48 h period before harvesting at E10.5.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We thank Yongqin Wu and Megumi Aita for performing the in situ hybridization and Amanda Peters, Matthew Fralish and Thomas Harrigan for technical assistance.

Conflict of Interest statement. There have been no influences that might bias the work reported in the results, figures, and tables, or modify any interpretations, implications or opinions stated in the Results and Discussion.

FUNDING

This work was supported by an American Heart Association (grant 0815067E to D.G.), National Institutes of Health (grants NICHHD042182 and NIMH64065 to A.-S.L.) as well as NINDS NS031768 to the University of North Carolina Neuroscience Center Expression Profiling and In Situ Hybridization core facilities, and S10RR025565 from the National Center for Research Resources for GWU Center for Microscopy Imaging and Analysis.

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