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RESEARCH ARTICLE

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Activation of WNT signaling restores the facial deficits in a zebrafish with defects in cholesterol metabolism

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Summary

Inborn errors of cholesterol metabolism occur as a result of mutations in the cholesterol synthesis pathway (CSP). Although mutations in the CSP cause a multiple congenital anomaly syndrome, craniofacial abnormalities are a hallmark phenotype associated with these disorders. Previous studies have established that mutation of the zebrafish *hmgcs1* gene (Vu57 allele), which encodes the first enzyme in the CSP, causes defects in craniofacial development and abnormal neural crest cell (NCC) differentiation. However, the molecular mechanisms by which the products of the CSP disrupt NCC differentiation are not completely known. Cholesterol is known to regulate the activity of WNT signaling, an established regulator of NCC differentiation. We hypothesized that defects in cholesterol synthesis are associated with reduced WNT signaling, consequently resulting in abnormal craniofacial development. To test our hypothesis we performed a combination of pharmaceutical inhibition, gene expression assays, and targeted rescue experiments to understand the function of the CSP and WNT signaling during craniofacial development. We demonstrate reduced expression of four canonical WNT downstream target genes in homozygous carriers of the Vu57 allele and reduced *axin2* expression, a known WNT target gene, in larvae treated with Ro-48-8071, an inhibitor of cholesterol synthesis. Moreover, activation of WNT signaling via treatment with WNT agonist I completely restored the craniofacial defects present in a subset of animals carrying the Vu57 allele. Collectively, these data suggest interplay between the CSP and WNT signaling during craniofacial development.

KEYWORDS
cholesterol, facial development, neural crest cells, WNT

1 | INTRODUCTION

The cholesterol synthesis pathway (CSP) produces two unique lipid molecules, cholesterol, an essential membrane component and precursor for steroids (Craig & Malik, 2020), and isoprenoids, a class of lipids essential for cell signaling (Lange, Rujan, Martin, & Croteau, 2000). Mutations that inhibit this pathway cause inborn errors of cholesterol synthesis. There are eight different inborn errors of cholesterol metabolism, each caused by mutations in a different enzyme of the CSP (Porter, 2002). These mutations cause a multiple congenital

anomaly syndrome; however, craniofacial abnormalities are a hallmark phenotype of these disorders (Porter, 2002). Multiple model systems have been developed in mice (Nwokoro, Wassif, & Porter, 2001) and zebrafish (Quintana, Hernandez, & Gonzalez, 2017, p. 1; Signore et al., 2016) to understand the mechanisms by which the products of the CSP regulate facial development.

The bone and cartilage of the face develops from neural crest cells (NCCs), a multipotent progenitor cell that arises from the dorsal end of the neural tube. A subset of NCCs, cranial NCCs (CNCC), migrate, proliferate, and differentiate into bone and cartilage structures of the face (Bhatt, Diaz, & Trainor, 2013). We and others have demonstrated that cholesterol is an essential mediator of CNCC differentiation and that the inhibition of cholesterol causes facial defects in zebrafish (Quintana et al., 2017; Signore et al., 2016). The molecular mechanisms underlying these differentiation defects are not completely known. However, cholesterol is known to regulate cell signaling in the form of lipid rafts and via the modification of specific proteins (Sheng et al., 2012). For example, cholesterol modification of sonic hedgehog (SHH) is essential for activation (Koleva, Rothery, Spitaler, Neil, & Magee, 2015).

Activation of SHH is essential for facial development and therefore, in previous studies we hypothesized that cholesterol regulates facial development via activation of SHH. However, mutation of the CSP in developing zebrafish causes defects in facial development in the absence of abnormal SHH signaling (Quintana et al., 2017). These data strongly suggest that the CSP regulates facial development in a SHH independent manner. Several other signaling cascades are essential for facial development, including FGF, BMP, WNT, and retinoic acid (Shimomura et al., 2019). Of these, cholesterol is required for activation of canonical WNT signaling via the PDZ domain in the dishevelled protein (Sheng et al., 2012). Therefore, we hypothesized that mutation of the CSP causes facial abnormalities due to defects in WNT activation.

To test this hypothesis, we performed a combination of loss of function, pharmacological inhibition assays, and restoration experiments using the developing zebrafish. The inhibition of cholesterol synthesis was associated with a significant decrease in the expression of canonical WNT downstream target genes and the expression of *sox10* and *col2a1a*, two markers of chondrocyte differentiation (Kelsh, 2006, p. 10) at 54 hours post fertilization (HPF). The activation of WNT using a pharmaceutical agonist completely restored the facial deficits present in approximately 50% of animals harboring the Vu57 allele. Collectively, these data suggest that cholesterol regulates facial development in a WNT dependent manner.

2 | MATERIALS AND METHODS

2.1 | Zebrafish care/embryo collection

Embryos were obtained by mating male and female pairs of Tupfel long fin, *hmgcs1*^{Vu57} (Mathews et al., 2014), *Tg*(*sox10*:memRFP; Kucenas et al., 2008), or *Tg*(*sox10*:tagRFP; Blasky, Pan, Moens, & Appel, 2014). Experiments were performed in all of the available lines harboring the Vu57 allele and combined in biological replicates to ensure the penetrance of the phenotype. Similar results were obtained regardless of strain or reporter background. Embryos were maintained in an incubator at 28 °C and all experiments were performed at The University of Texas at El Paso according to approved guidelines from the Institutional Animal Care and Use Committee (IACUC) protocol 811689-5.

2.2 | Genotyping

Genotyping was performed as described in (Quintana et al., 2017) and (Mathews et al., 2014). DNA was extracted from all samples using phenol: chloroform ethanol precipitation as described (Quintana et al., 2017). Samples were collected either by clipping the fins of adult zebrafish, collecting eggs, or dissecting the embryos and using a small piece of tissue for DNA extraction. DNA was extracted in DNA extraction buffer (10 mM Tris pH 8.2, 10 mM EDTA, 200 mM NaCl, 0.5% SDS, and 200 μg/ml proteinase K) for 3 hr at 55 °C. Polymerase chain reaction (PCR) was used to amplify the target sequence of the *hmgcs1* gene. PCR and restriction digest was performed as previously described (Quintana et al., 2017) (Hernandez, Castro, Reyes-Nava, Montes, & Quintana, 2019). For quantitative real time PCR (qPCR) analysis, embryos/larvae were anesthetized and a small clip of the tail was taken for downstream genotyping while the remaining part of the embryo was lysed individually in Trizol (Fisher). Upon genotyping results, the heads from each genotype were pooled together, RNA was isolated, and subsequent qPCR was performed.

2.3 | RNA extraction and in situ hybridization

RNA isolation was performed with Trizol according to manufacturer's protocol (Fisher). Total RNA (100–500 ng) was reverse transcribed using the Verso cDNA synthesis kit (Fisher) and cDNA was used for qPCR. qPCR was performed as described (Quintana et al., 2017). Primers for qPCR are as follows: (a) *axin2 fwd* 5′-CAACC AAGCACATCCATCAC-3′ and *rev* 5′-TCCATGTTCACCTCCTCTCC-3′, (b) *rpl fwd* 5′-TCCCAGCTGCTCTCAAGATT-3′ and *rev* 5′-TTCTT GGAATAGCGCAGCTT-3′, (c) *sox10 fwd* 5′-ACGCTACAGGTCAGA GTCAC- 3′ and *rev* 5′-ATGTTGGCCATCACGTCATG-3′, (d) *edn1 fwd* 5′-CAAACACAAGCTGGCAGAAA-3′ and *rev* 5′-CGTGTGCAGTGA ATGAGCTT-3′, (e) *ccnd1 fwd* 5′ CTGTGCGACAGACGTCAACT-3′ and *rev* 5′-CTGACACGATCGCAGACAGT-3′, (f) *lef1 fwd* 5′ ACCCACA GGTGAAACAGGAG-3′ and *rev* 5′-GGCCGAGGATCTGATTGATA-3′, and (g) *col2a1a fwd* 5′- GTGTGTGATTCGGGGACTGT-3′ and *rev* 5′-TTTGCACCAAGTGACCCGAT-3′. Analysis was performed at 30 HPF or 54 HPF as indicated in the figure legend.

2.4 | Drug treatments

Wnt agonist-1 was dissolved in 100% DMSO and embryos were treated with a final concentration ranging from 0.1–1.0 μM. Concentrations were developed from recommendations previously described

(Liu et al., 2005). Final concentration of DMSO was less than or equal to 0.01% in all treated samples and the vehicle control treatment. Wnt agonist-1 treatment was initiated at 30 HPF, consistent with the onset of facial defects (Quintana et al., 2017) and removed at 54 HPF, for a total treatment time of no more than 24 hrs at 28 °C. Following treatment, embryos were maintained at 28 °C until 4 days post fertilization (DPF) in system water. For all other drug treatments, each drug was added at the indicated concentration at the sphere stage as previously described (Hernandez et al., 2019; Quintana et al., 2017; Quintana, Picchione, Klein Geltink, Taylor, & Grosveld, 2014). Embryos were treated with 1, 1.5, 2, or 3 μM Ro 488071 (Santa Cruz Biotechnology), 8 μM lonafarnib (Millipore-Sigma), or 2 μM atorvastatin (Millipore-Sigma); all concentrations were empirically derived and previously published and validated (Hernandez et al., 2019; Quintana, Picchione, et al., 2014, p. 7; Quintana et al., 2017). All larvae were harvested at 30 HPF or 54 HPF in each treatment group for qPCR analysis.

2.5 | Staining and imaging

Staining was performed as described in Quintana, Geiger, et al. (2014). Briefly, embryos were harvested at 4 DPF and fixed in a 2% PFA and 7.4 pH PBS solution for 1 hr at room temperature (RT). After fixing, embryos were stained with Alcian Blue (Anatech Ltd., #862 5 ml 0.4% Alcian Blue in 70% EtOH, 5 ml Tris pH 7.5, 0.5 ml 1 M MgCl2, 36 ml EtOH 80%) overnight at RT. Samples were washed with 80% EtOH/100 mM Tris pH 7.5/10 mM $MgCl_2$ for 5 min, 50% EtOH/100 mM Tris pH 7.5 for 5 min, and 25% EtOH/100 mM Tris pH 7.5 for 5 min, each at RT. Pigment was removed by incubation in bleach buffer (100 μl 30% H2O2, 25 μl 20% KOH, 875 μl H_2O) for 10 min at RT. Bleach solution was removed by performing 2–10-min washes of 25% glycerol/0.1% KOH.

3 | RESULTS

3.1 | Inhibition of cholesterol synthesis reduces expression of WNT downstream target genes

We have previously demonstrated that inhibition of cholesterol synthesis causes craniofacial abnormalities and decreased expression of *sox10*, a marker of CNCCs at 3 and 4 DPF (Kelsh, 2006, p. 10). However, the mechanisms by which cholesterol regulates facial development are unknown because SHH activity is normal at 2 DPF in carriers of the Vu57 allele. Based on published data, we hypothesized that defects in cholesterol synthesis disrupt WNT signaling, consequently resulting in facial dysmorphia. To test this hypothesis, we used qPCR to monitor the expression of four downstream target genes of the canonical WNT signaling cascade including *lef1*, *edn1*, *axin2*, and *ccnd1* (Herbst et al., 2014) in animals carrying the Vu57 allele. We analyzed the expression of each gene at 30 HPF. We chose 30 HPF because previous studies have demonstrated that CNCCs are normal in the Vu57 allele at this time point and therefore, we hypothesized that defects in WNT signaling should precede the craniofacial abnormalities associated with the Vu57 allele. As shown in Figure 1a, homozygous carriers of the Vu57 allele had a statistically significant decrease in the expression of *axin2*, *lef1*, and *ccnd1* at 30 HPF. We observed decreased expression of *edn1*, however it was not statistically significant across two biological replicates, with each replicate containing a pool of eight embryos per group for a total N of 16 across biological replicates.

3.2 | Decreased WNT activation is cholesterol dependent

Mutation of *hmgcs1* disrupts the synthesis of cholesterol and isoprenoids, two products of the CSP. We have demonstrated in previous studies that the independent inhibition of either product is associated with craniofacial defects (Quintana et al., 2017). Moreover, isoprenoids are known to regulate cell signaling and therefore, we surmised that the abnormal expression of WNT downstream target genes could be the result of abnormal cholesterol synthesis, abnormal isoprenoid synthesis, or both. Importantly, inhibition of isoprenoid synthesis causes a more severe facial phenotype than inhibition of cholesterol synthesis (Quintana et al., 2017). Therefore, we treated wild type embryos with (a) 1.5 μM Ro 48-8071 (4′-[6-(allylmethylamino)hexyloxy]-4-bromo-2′-fluorobenzophenone fumarate) to inhibit cholesterol, (b), 8 μM lonafarnib to inhibit isoprenoids, or (c) 2 μM atorvastatin, which inhibits the synthesis of both cholesterol and isoprenoids and then measured the expression of *axin2* by qPCR at 30 HPF. Treatment with either atorvastatin or Ro 48-8071 resulted in a statistically significant decrease in *axin2* expression (Figure 1b). However, treatment with lonafarnib, which inhibits farnesylated isoprenoids did not result in a statistically significant decrease in *axin2* expression. We next analyzed the effects of increased concentrations of Ro 48-8071 on *axin2* expression. Embryos were treated with 1, 2, or 3 μM Ro 48-8071 and the level of *axin2* expression was measured by qPCR at 30 HPF. We observed decreased *axin2* expression in all groups, with the most dramatic decrease in embryos treated with the highest concentration of 3 μM (Figure 1c). *axin2* expression was utilized as a global marker of WNT activation and not as a direct mediator of CNCC differentiation because *axin2* is not highly expressed in the pharyngeal arches of developing embryos at 24–30 HPF, but more specifically expressed in the dorsal central nervous system from midbrain to tail, tail bud, and part of the optic stalks (Sprague et al., 2008).

3.3 | Early CNCCs are normal, but *sox10* expression is decreased during early development

We have previously demonstrated that homozygous carriers of the Vu57 allele have normal early development of CNCCs as detected by *dlx2a* expression (1 DPF) and *sox10* expression (18 somites) (Quintana

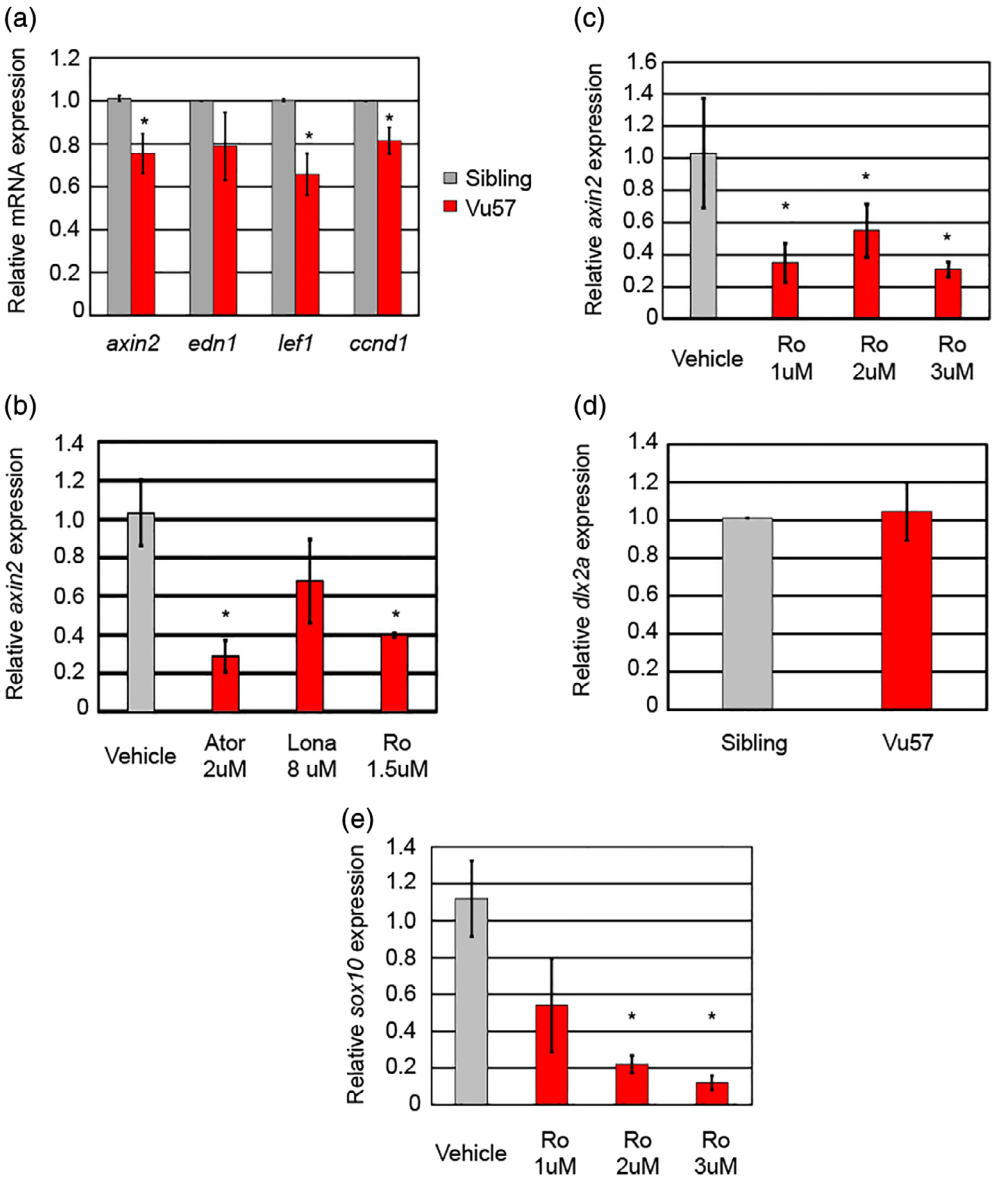

FIGURE 1 Inhibition of cholesterol synthesis is associated with decreased expression of WNT target genes. (a) RNA was isolated from homozygous carriers of the Vu57 allele (Vu57) or wild type siblings (Sibling) at 30 hours post fertilization (HPF) and the relative expression of *axin2*, *edn1*, *lef1*, *and ccnd1* were measured by quantitative real time PCR (qPCR). *N* = 8/group in two biological replicates for a total *N* of 16. **p* < .05. (b) Wild type larvae were treated with vehicle control (DMSO), 2 μM atorvastatin (ATOR), 8 μM lonafarnib (lona), or 1.5 μM Ro-48-8071 at sphere stage. Total RNA was isolated from a pool of embryos (*N* = 14) and the relative expression of *axin2* was measured by quantitative real time PCR (qPCR). (c) Embryos were treated with vehicle control (DMSO), 1, 2, or 3 μM Ro 48-8071 at the sphere stage and total RNA was isolated at 30 HPF from a pool of embryos (*N* = 30) and the relative expression of *axin2* was measured by qPCR. **p* < .05. Error bars represent *SD*. (d). RNA was isolated from homozygous carriers of the Vu57 allele or Sibling wild type at 30 HPF and the relative expression of *dlx2a* was measured using qPCR. *N* = 8/group with two biological replicates for a total *N* = 16. Error bars represent *SD*. (e) *sox10* expression was monitored in embryos treated with vehicle control (DMSO), 1, 2, or 3 μM Ro 48-8071 at the 30 HPF (*N* = 30). Error bars represent *SD*. **p* < .05

et al., 2017). We sought to confirm that early CNCC development is normal despite defects in WNT signaling at 30 HPF. To do so, we measured the expression of *dlx2a* at 30 HPF using qPCR. We did not detect defects in *dlx2a* expression, consistent with our previously published studies (Figure 1d; Quintana et al., 2017). Moreover, *sox10* is an early neural crest marker, but is down regulated in CNCCs. Despite this down regulation, *sox10* expression persists in other NCC subsets and its expression is regulated by WNT signaling (Aoki et al., 2003). Thus, we hypothesized that defects in cholesterol synthesis would cause a decrease in *sox10* expression as early as 30 HPF. To test this, we treated wild type larvae with 1, 2, or 3 μM Ro48-8071 and measured the expression of *sox10* using qPCR. We observed a concentration dependent decrease in *sox10* expression (Figure 1e). These data suggest that the absence of WNT signaling

affects NCC development, however, early CNCC development is normal at 30 HPF as these cells down regulate *sox10* until late stage chondrogenesis, when Sox10 is re-up regulated and contributes to the activation of *col2a1a* (Suzuki, Sakai, Osumi, Wada, & Wakamatsu, 2006).

3.4 | Activation of WNT signaling restores the facial deficits in the Vu57 allele

We hypothesized that activation of WNT signaling might restore the facial phenotypes present in the Vu57 allele. To test this, we treated offspring of the Vu57 allele with Wnt-Agonist 1. We initiated treatment at 30 HPF because we demonstrated that the expression of WNT downstream target genes are reduced at 30 HPF, yet CNCCs are normal according to *dlx2a* expression (Figure 1). In addition, previous studies have demonstrated that cholesterol deficiency specifically inhibits late stage CNCC differentiation, with little to no effects on early CNCC specification and migration (Quintana et al., 2017; Signore et al., 2016). We removed the treatment at 54 HPF because we have previously shown CNCC deficits at 3 DPF and because prolonged treatment with WNT-agonist 1 caused necrosis in the head, pigment defects, and inhibited hatching from the chorion. After completion of the treatment, larvae were either sacrificed for mRNA expression (54 HPF) or grown to 4 DPF in media without drugs before being assayed for cartilage development with Alcian blue (Figure 2a). As previously described, vehicle control treated homozygous carriers of the Vu57 allele had significant craniofacial defects including the complete loss of the Meckel's cartilage, ceratohyal, and ceratobranchial cartilages (Figure 2b,c). In contrast, treatment with 0.1 μM Wnt-agonist 1 restored the facial defects to wild type levels (Figure 2e; $p < .0001$). We observed a complete restoration of all viscerocranium elements (Meckel's cartilage, palatoquadrate, ceratohyal, basihyal, and ceratobranchial cartilages) in 16/30 treated mutants, which was statistically significant using a Fisher's Exact Test (Table 1). Only those animals with restoration of all elements of the viscerocranium were counted toward the rescue and partial restoration was not considered because the facial phenotypes present in the Vu57 allele are heterogeneous with varied severity. Therefore, we only counted individuals with full restoration in our final quantification. Importantly, we were limited to a concentration of 0.1 μM in our analysis of cartilage restoration as higher concentrations were associated with brain necrosis and failure to hatch from the chorion (0.2 μM beyond 54 HPF) or death (0.75 and 1.0 μM). As shown in Table 1, some heterozygous individuals demonstrated with craniofacial abnormalities, but this phenotype is not completely penetrant and was found in our previous study not to be statistically significant (Quintana et al., 2017).

In addition, we measured the expression of *sox10*, *axin2*, and *col2a1a* in treated and untreated larvae. We performed our analysis of gene expression at 54 HPF upon removal of the WNT agonist drug. We analyzed expression at 54 HPF because we hypothesized the effects of the drug would be the highest immediately after treatment (Figure 2a). Importantly, we were able to analyze expression in 0.1 and 0.2 μM treatment groups because larvae did not die from significant negative effects at 54 HPF. Based on qPCR analysis, we observed reduced expression of *axin2*, *sox10*, and *col2a1a* in homozygous carriers of the Vu57 allele (Figure 2f, red bars). In contrast, homozygous Vu57 larvae treated with 0.1 μM WNT agonist-1 had increased expression of *sox10*, *col2a1a*, and *axin2* to either wild type levels or above wild type levels (Figure 2f, yellow bars), indicative of a restoration of the craniofacial and WNT defects present in mutant larvae. These effects were concentration dependent because treatment with 0.2 μM WNT agonist-1 increased the expression of *col2a1a*, *sox10*, and *axin2* well above wild type levels (Figure 2f, blue bars). These data are consistent with the 50% of individuals rescued at the level of cartilage deposition using the 0.1 μM concentration (Figure 2b–e). Most importantly, restoration of *col2a1a* expression is indicative of restored chondrogenesis, as *col2a1a* is expressed in differentiating chondrocytes (Simões-Costa & Bronner, 2015).

4 | DISCUSSION

We have previously demonstrated that mutation of the zebrafish *hmgcs1* gene (the Vu57 allele) causes craniofacial defects that are caused by defects in late stage NCC differentiation (Quintana et al., 2017). These data are supported by other zebrafish studies analyzing the craniofacial defects present upon mutation of *hmgcr* (Signore et al., 2016). The onset of these late stage NCC deficits occur after 30 HPF, as Signore and colleagues demonstrated that treatment with statins at 30 HPF was sufficient to induce craniofacial abnormalities. Despite these advances, a molecular mechanism by which facial abnormalities develop in animals with reduced cholesterol synthesis has not been characterized. Multiple studies have suggested that defects in cholesterol synthesis are related to altered SHH signaling (Shin et al., 2019), however homozygous Vu57 mutants do not show altered SHH signaling during early CNCC development (Quintana et al., 2017).

Cholesterol is required for the activation of various cell signaling cascades (Shimomura et al., 2019), including SHH and WNT, both of which are required for craniofacial development (Shimomura et al., 2019; Shin et al., 2019). For example, WNT activation requires the activity of dishevelled, a scaffolding protein (Bernatik et al., 2011). The dishevelled protein requires cholesterol modification of its PDZ domain for proper activation (Sheng et al., 2014). Based upon these data, we hypothesized that defects in cholesterol synthesis would decrease WNT activation. Consistent with this hypothesis, we observed decreased activation of four downstream canonical WNT target genes. Our analysis focused on the expression of only four WNT target genes, each of which with unique expression patterns that may or may not be restricted to CNCC development. Instead, we used their expression to establish defective WNT activation globally. Future experiments that identify the level of WNT activation or the phosphorylation status of β-catenin in CNCCs are required. While our data does not address cell autonomy, we do show that globally WNT

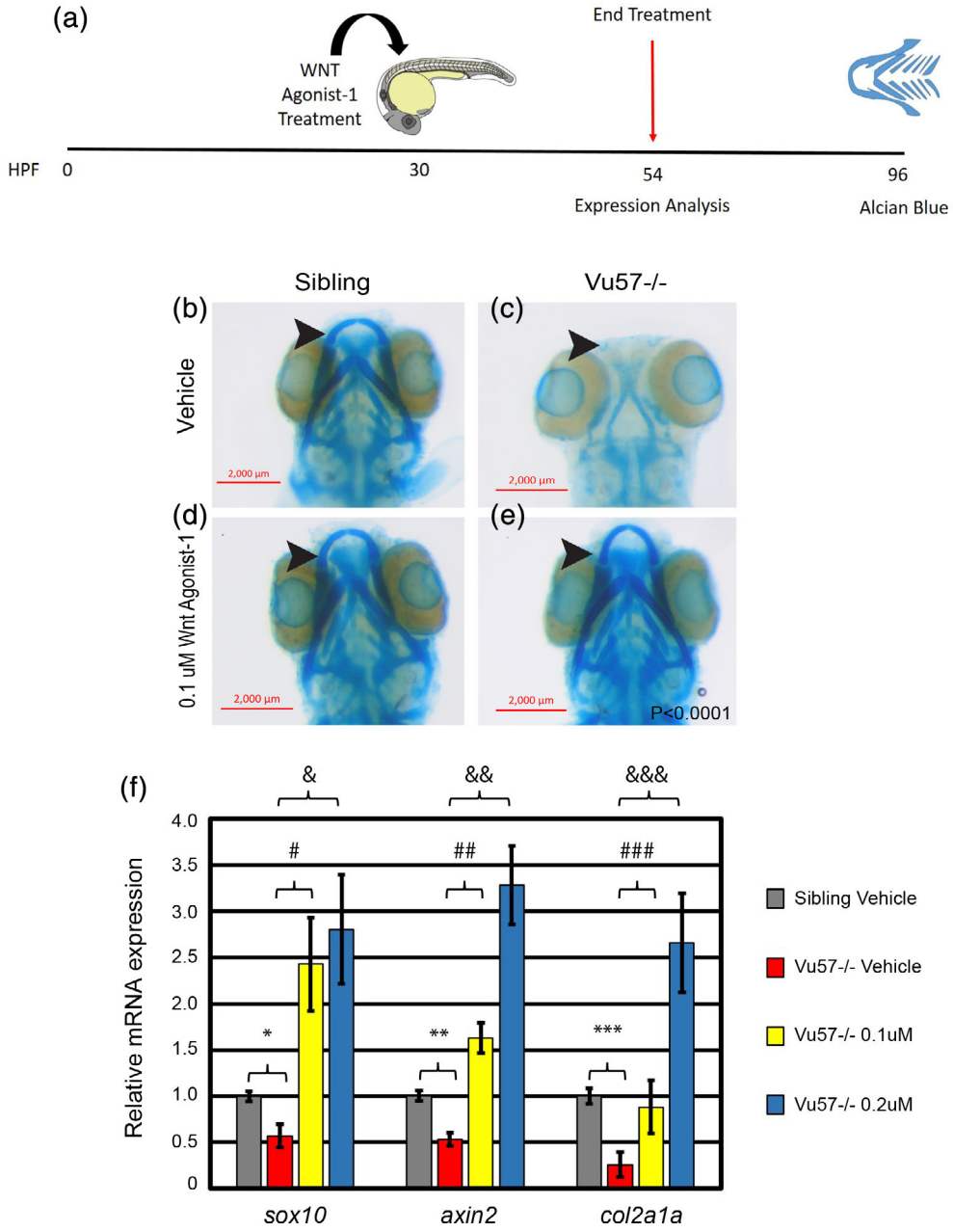

FIGURE 2 Activation of WNT signaling restores the facial defects present in the Vu57 allele. (a) Experimental design schematic with onset of treatment with WNT-Agonist I at 30 hours post fertilization (HPF) and removal of treatment at 54 HPF. Alcian Blue was performed at 4 days post fertilization (DPF) in treated and untreated individuals that were incubated in embryo media from 54–96 HPF following treatment. (b–e) Alcian blue staining was performed at 4 DPF in homozygous carriers of the Vu57 allele (Vu57−/−) and wild type siblings (Sibling) according to the treatment schematic in (a). Total numbers of animals reflected in Table 1. *p = .0001 using a Fisher's Exact test. (f). Homozygous carriers of the Vu57 allele (Vu57−/−) or wild type siblings (Sibling) were treated with vehicle control (DMSO) or WNT-agonist I at either 0.1 or 0.2 μM concentration according to the schematic in (a). Total RNA was isolated from a pool of embryos and the expression of *sox10*, *col2a1a*, or *axin2* was measured by quantitative PCR (qPCR). Error bars represent *SD* of biological replicates. N = 11/group except for 0.2 μM concentration where N = 9. *p = 4.07457E-05, **1.14579E-06, ***p = 3.80572E-07, #2.45716E-05, ##p = 7.28315E-08, ###p = .000716073, &p = 3.22846E-05, &&p = 7.2866E-07, &&&p = 8.45279E-07

signaling is reduced. Our work is supported by previous studies demonstrating that cholesterol deficient induced pluripotent stem cells (iPSCs) have reduced WNT signaling (Francis et al., 2016). However, these studies were performed in the context of mutations in *DHCR7* that encodes an enzyme farther down in the CSP, an important distinction, because mutations in this gene inhibit cholesterol synthesis, but not isoprenoids. A primary difference between this study and our study is the mechanism by which mutation of *DHCR7* affects WNT activity. For example, this mutation causes the accumulation of a specific metabolite (7-dehydrocholesterol), which is responsible for

TABLE 1 Presence of facial abnormalities after treatment with WNT-Agonist I in the Vu57 allele

Treatment	Wild type (WT)	# WT affected	Heterozygous (HET)	# HET affected	Homozygous mutant (MT)	# MT affected	Total embryos	Percent survival (%)
None	14	0/14	25	6/25	17	17/17	56	100
DMSO	25	0/25	35	5/35	25	25/25	85	100
WNT 0.1 μM	15	0/15	32	7/32	30	16/30	77	100

Note. Craniofacial phenotypes in siblings of the *hmgcs1*^{vu57} allele (wild type), heterozygous carriers, and homozygous carriers treated with vehicle control (DMSO) or treatment with WNT agonist-1 (WNT).

defects in WNT signaling. We do not yet know whether mutation of *hmgcs1* causes accumulation of metabolites. Interestingly, treatment with Ro-48-8071, which inhibits the *LSS* enzyme would more closely resemble the data from Francis and colleagues. Thus, our analysis cannot rule out whether the defects we observe in WNT signaling are a consequence of the accumulation of metabolites or loss of cholesterol. Future experiments that monitor the level of cholesterol modification of dishevelled or the accumulation of metabolites are warranted. Despite this gap in our understanding, it is clear that activation of WNT signaling with WNT agonist-1 can restore the facial phenotypes present in a significant number of animals carrying the Vu57 allele. Thus, whether autonomous or non-autonomous, our data suggests that the CSP regulates facial development in a WNT dependent manner.

We have previously demonstrated that isoprenoids regulate facial development in a cholesterol independent manner (Quintana et al., 2017). In fact, inhibition of isoprenoid synthesis resulted in a more severe facial phenotype than inhibition of cholesterol synthesis. In addition, isoprenoids are necessary for protein prenylation and cell signaling (Lange et al., 2000) and are known to affect several signaling pathways including WNT (Robin et al., 2014). Based upon these data, we tested whether inhibition of cholesterol or isoprenoids affected WNT signaling. We found that inhibition of cholesterol was associated with decreased *axin2*, however, inhibition of isoprenoid synthesis did not statistically reduce the expression of *axin2*. Treatment with atorvastatin caused a significant reduction in *axin2*, whereas treatment with Ro 48-8071 was more moderate. While these data suggest isoprenoid synthesis is not required for WNT activation, the degree to which isoprenoid deficiency contributes to WNT suppression remains unclear. However, our results suggest there are at least some cholesterol dependent mechanisms regulating WNT activity.

Our most significant finding is that the facial phenotypes associated with the Vu57 allele can be restored to wild type levels after treatment with WNT agonist 1. WNT agonist 1 stabilizes β-catenin and leads to WNT activation (Liu et al., 2005). We noted that a concentration of 0.1 μM WNT agonist 1 was sufficient to restore the facial phenotypes in 50% of homozygous mutants. This restoration is accompanied by increased *sox10* and *col2a1a* expression, which is concentration dependent. Of importance is the finding that only 50% of the treated animals were rescued. Given that the Vu57 allele abrogates both cholesterol and isoprenoid synthesis and that each of these lipids independently regulate facial development, we hypothesize that any animals not demonstrating a full rescue is a consequence of deficiencies in isoprenoid synthesis.

Here, we demonstrate that inhibition of the CSP is associated with decreased expression of WNT downstream target genes. Moreover, our work demonstrates that activation of WNT in larvae harboring the Vu57 allele completely restores the craniofacial defects present in a subset of homozygous mutants. These data implicate cholesterol as a key component of WNT signaling and craniofacial development.

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CONFLICT OF INTEREST

Authors report no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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