Inhibition of the 3-Hydroxy-3-Methyl-Glutaryl-CoA Reductase Induces Orofacial Defects in Zebrafish

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Background: Orofacial clefts (OFCs) are common birth defects, which include a range of disorders with a complex etiology affecting formation of craniofacial structures. Some forms of syndromic OFCs are produced by defects in the cholesterol pathway. The principal enzyme of the cholesterol pathway is the 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR). Our aim is to study whether defects of HMGCR function would produce orofacial malformation similar to those found in disorders of cholesterol synthesis. **Methods:** We used zebrafish *hmgcrb* mutants and HMGCR inhibition assay using atorvastatin during early and late stages of orofacial morphogenesis in zebrafish. To describe craniofacial phenotypes, we stained cartilage and bone and performed *in situ* hybridization using known craniofacial markers. Also, we visualized neural crest cell migration in a transgenic fish. **Results:** Our results showed that mutants displayed loss of cartilage and diminished orofacial outgrowth, and in some cases palatal cleft. Late treatments with statin show a similar phenotype. Affected-siblings displayed a moderate phenotype, whereas early-treated embryos had a minor cleft. We found reduced expression of the downstream component of Sonic Hedgehog-signaling *gli1* in ventral brain, oral ectoderm, and pharyngeal endoderm in mutants and in late atorvastatin-treated embryos. **Conclusion:** Our results suggest that HMGCR loss-of-function primarily affects postmigratory cranial neural crest cells through abnormal Sonic Hedgehog signaling, probably induced by reduction in metabolites of the cholesterol pathway. Malformation severity correlates with the grade of HMGCR inhibition, developmental stage of its disruption, and probably with availability of maternal lipids. Together, our results might help to understand the spectrum of orofacial phenotypes found in cholesterol synthesis disorders.

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Key words: HMGCR; orofacial malformation; orofacial cleft; cholesterol pathway; SHh-signaling; statins; zebrafish

Introduction

Craniofacial malformations comprise more than 30% of human congenital anomalies (Wilkie and Morris-Kay, 2001), making orofacial clefts (OFCs) one of the most common craniofacial defects with a complex etiology (Dixon et al., 2011; Setó-Salvia and Stanier, 2014). Approximately 70% of the OFCs are nonsyndromic occurring as an isolated condition, apparently without any other structural or cognitive abnormality. The remaining 30% of OFCs are syndromic (Dixon et al., 2011), which also display other congenital anomalies such as neurological abnormalities (holoprosencephaly, microcephaly, etc.), cardiac defects (atrial and ventricular septa dysgenesis), syndactyly, polydactyly, and intellectual disability (reviewed in Moebius et al., 2000; Porter and Herman, 2011).

Of interest, mutations in genes encoding enzymes of the postsqualene mevalonate (MVA) pathway cause at least eight human malformation syndromes. Four of these present multiple congenital malformations that include orofacial clefts: (i) the Smith-Lemli-Opitz syndrome (SLOS) produced by mutations in the *DHCR7* gene and in increments in 7-dehydrocholesterol (7DHC) levels, and SLOS-like syndromes; (ii) desmosterolosis caused by mutations in *DHCR24*; (iii) the X-linked dominant chondrodysplasia puctata (CDPX2), related to alterations in enhancer binding protein; and (iv) lathosterolosis, produced by missense mutations in the *SC5D* gene. Depending on which enzyme is disrupted, either abnormal accumulation of sterols and/or deficit of cholesterol and oxysterols have been proposed as being responsible for OFC induction (Moebius et al., 2000; Porter and Herman, 2011).

In the MVA pathway, HMGCR catalyzes the rate-limiting step in the synthesis of isoprenoids, sterols, and cholesterol (Espenshade and Hughes, 2007). Isoprenoids are essential for prenylation of small GTPases (Nguyen et al., 2009). Moreover, cholesterol is attached to Hedgehog (Hh) proteins and is necessary for its proper signaling (Porter et al., 1996; Lewis et al., 2001). Both families of proteins

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are essential for craniofacial morphogenesis (Cordero et al., 2004; Wada et al., 2005; Eberhart et al., 2006; Kerr et al., 2008; Schwend and Ahlgren, 2009; Thomas et al., 2010; Liu et al., 2013; Heyne et al., 2015). For instance, in vitro studies have shown that sterol/oxysterol levels modulate Sonic Hedgehog (SHh) signaling (Cooper et al., 2003; Corcoran and Scott, 2006; Dwyer et al., 2007; Rohatgi et al., 2007). Nevertheless, only a few reports have demonstrated that cholesterol metabolism can modulate proper activation of the Hh signaling pathway in vivo.

Even though it is known that cholesterol metabolism, SHh signaling, and craniofacial development are interconnected processes, the mechanistic relationship between a defective cholesterol biosynthetic pathway and the appearance of craniofacial malformations is still unclear. It has been proposed that OFCs are more likely produced by increased sterols precursors than by deficit of cholesterol. For instance, Insig-1 and Insig-2 proteins are required for regulation of cholesterol synthesis and *insig-1/insig-2* double-knockout mice (*Insig*-DKO) exhibit facial defects ranging from a cleft palate to complete facial cleft with normal cholesterol levels and over-accumulation of sterols. However, this study did not show a direct relationship between sterols, cleft, and SHh signaling defects (Engelking et al., 2006). Also, defects in another enzyme of postsqualene MVA pathway, the 17β-hydroxysteroid dehydrogenase (17β-HSD7), lead to an impaired response to SHh pathway due to the accumulation of specific sterols with normal levels of cholesterol. As a result, abnormalities in central nervous system and disrupted appendicular skeletal morphogenesis are observed without defects in craniofacial structures (Stottmann et al., 2011).

Conversely, cyclodextrin-induced lipid and sterol depletion in chick embryos results in variable loss of the frontonasal process and diverse midline defects (Cooper et al., 2003). Furthermore, a recent study has shown that, in SLOS, defects in SHh signaling are more due to the cholesterol deficit than to the oxysterol reduction or 7DHC accumulation (Blassberg et al., 2016). Whether deficiency in cholesterol and in intermediate metabolites of the MVA pathway also can induce craniofacial malformation remains an important question that has not yet been resolved. Studying effects on the craniofacial development of the *hmgcr* mutation, which should genetically block the cholesterol pathway, would be very informative in this regard. However, the *Hmgcr* mutation is lethal during early embryonic development in mice (Ohashi et al., 2003). Thus, the putative role of HMGCR in craniofacial abnormal formation and OFC induction remained largely uninvestigated.

In this work, we study the impact of *hmgcr* loss on orofacial development in zebrafish *(Danio rerio)* because, in contrast to mice, the mutation of this enzyme is not lethal during early development (D'Amico et al., 2007). In past years, zebrafish has become a powerful model for the study of craniofacial morphogenesis and OFC etiology (Wada et al., 2005; Swartz et al., 2011; Dougherty et al., 2013). The *hmgcrb* mutation in zebrafish resulted in thicker yolk extension, kinked notochord, and heart-tube development defects (D'Amico et al., 2007), but craniofacial anomalies have not been assessed in this mutant.

There are two orthologues of *hmgcr* in zebrafish, *hmgcra* and *hmgcrb*, and a mutant of the second paralogous is available (D'Amico et al., 2007). Moreover, loss-of-function of *hmgcra* by antisense morpholino injection promoted muscle fiber damage, but orofacial defects were not observed (Hanai et al., 2007). Also, this model is suitable for temporally restricted inhibition assays using pharmacological compounds such as statins (Thorpe et al., 2004; D'Amico et al., 2007; Eisa-Beygi et al., 2013). This type of study enables control of the temporal window of pharmacological inhibition to study the specific impact of the enzymatic impairment on different phases of developmental processes.

We have exploited the advantages of the zebrafish to study whether defects of HMGCR function would induce craniofacial anomalies. We analyzed orofacial morphology and the expression of craniofacial genetic markers in the *hmgrb*$^{s617/s617}$ mutant line and in embryos treated with atorvastatin, a known inhibitor of the synthesis of isoprenoids, sterols, and cholesterol (Istvan and Deisenhofer, 2001), during neural crest cell (NCC) migration toward pharyngeal arches (early stage of craniofacial morphogenesis) and during chondrogenesis stages (late stage of orofacial development).

We found that *hmgcrb* mutants and atorvastatin-treated embryos during late stages of craniofacial morphogenesis had severe shortening of craniofacial structures, loss of cartilage elements, and OFCs. Conversely, HMGCR activity inhibition during early stage of orofacial morphogenesis caused minor defects. Thus, our results suggest that the HMGCR disruption perturbed mainly late stages of craniofacial development, such as condensation of cranial NCCs (CNCCs) in pharyngeal arches and chondrogenesis phases. Importantly, we found reduction of *gli1* expression, a downstream components of SHh signaling, in ventral brain, oral ectoderm, and pharyngeal endoderm of embryos with abnormal HMGCR function. Together, our results support that orofacial malformations caused by defects in the MVA pathway may be induced by a combined reduction of cholesterol, sterols, and isoprenoids, and defects in SHh pathway.

Materials and Methods

FISH LINES AND CARE

For this study, we used the following Zebrafish (*Danio rerio*) lines: wild-type Tübingen (WT), *Tg(-4.9 sox10::GFP)*^{ba2} (Wada et al., 2005), and *hmgrcb*$^{s617/s617}$ mutants (D'Amico et al., 2007). Embryos were obtained by

natural spawning, raised at 28.5 °C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$), and staged according to morphology (Kimmel et al., 1995) and age in hours postfertilization (hpf) and days postfertilization (dpf). The allele *s617* carries a point mutation (G to A) at 1575 position producing a change from Gly to Asp at codon 497. This glycine residue is highly conserved within a domain required for homodimerization or tetramerization of HMGCR (D'Amico et al., 2007). These lines are available at the Fish Facility of the ICBM, Faculty of Medicine, University of Chile. All experimental protocols were approved by the Bioethical Review Board on Animal Research, Faculty of Medicine, University of Chile.

WHOLE-MOUNT RNA *IN SITU* HYBRIDIZATION AND CARTILAGE STAINING

In situ hybridization was performed according to a standard protocol (Thisse and Thisse, 2008). The following probes were used: *sox10* (Dutton et al., 2001), *dlx2a* (Akimenko et al., 1994), *shha* (Krauss et al., 1993), *gli1* (Karlstrom et al., 2003), and *col2a1* (Yan et al., 1995). Cartilage and bone staining were performed as previously described (Walker and Kimmel, 2007). Images were captured using Nikon SMZ1000 and Nikon Eclipse 80i microscopes mounted with a Nikon Digital Sight DS-Fi1 digital camera connected to a computer running an image capture software (NIS-Elements F3.0). Contrast and brightness were adjusted using Adobe Photoshop CS4 software for better presentation.

PHARMACOLOGICAL INHIBITION OF HMG-COA REDUCTASE ASSAY

To inhibit HMGCR activity in a temporally restricted manner either at early or late stages of craniofacial morphogenesis, embryos were treated with atorvastatin (a lipophilic statin) (Sigma) according to standards protocols (Thorpe et al., 2004; D'Amico et al., 2007). Atorvastatin is a known competitive inhibitor of HMGCR that leads to the reduction of isoprenoids, sterols, and cholesterol (Istvan and Deisenhofer, 2001). The early inhibition assay (during NCCs migration to facial primordial) was carried out raising WT and *Tg(-4.9sox10::GFP)*^{ba2} embryos until 10 hpf and soaking them overnight in 5 μM atorvastatin/0.5% dimethylsulfoxide (DMSO) (Sigma) in E3 medium at 28.5 °C. In the late inhibition assay (during chondrogenesis), WT embryos were exposed to 5 μM atorvastatin/0.5% DMSO in E3 between 30 hpf and 48 hpf. In each case, control experiments were performed using the same protocol of the corresponding inhibition assay and exposing embryos to 0.5% DMSO in E3.

Embryos were fixed in 4% paraformaldehyde (Polysciences)/phosphate buffered saline buffer at 24, 48, 52, and 54 hpf (early inhibition assay), at 48, 52, and 54 hpf (late inhibition assay) and at 10, 14, 24, 48, 52, and 54 hpf (mutant line) for *in situ* hybridization or at 4.5 dpf for cartilage/bone staining for all conditions. Confocal microscopy analyses of early atorvastatin-treated transgenic embryos (10 per condition) were performed at 24 hpf.

CRANIOFACIAL STRUCTURES ANALYSIS

For morphological analyses of craniofacial structures, an extended focus projection from z-stack images of cartilage/bone staining was used. We took measurements from the images using ImageJ software. Three points of interest were measured: the angle between the cerathohyal and the midline point of the posterior cerathohyal junction with the basihyal; the length of the ethmoid (midline point of the most proximal anterior and distal posterior of the ethmoid); and the length of the cerathohyal (right and left lateral most point of the cerathohyal and right and left anterior–lateral junction of cerathohyal with the basihyal). Data were obtained measuring 8 to 10 embryos for each experimental condition.

CONFOCAL IMAGING

Images of green fluorescent protein (GFP) expression in *Tg(-4.9sox10::GFP)*^{ba2} embryos were acquired using a Leica TCS LSI confocal microscope equipped with 5 × objective and the super Z-zoom system Z6 APO A from 0.57 × to 3.6 × magnification. Embryos were fixed in 4% paraformaldehyde/phosphate buffered saline buffer/0.25% glutaraldehyde (Fluka) and mounted in 1% low melting point agarose. Images were captured with z = 0.5 μm, and confocal projections were obtained using Volocity software (Improvision).

STATISTICS ANALYSIS

Statistical analyses were performed using Mann-Whitney test for nonparametric data (OriginPro8 Software), and $p < 0.05$ were considered as significant. Data shown in Figures 1 and 2 correspond to mean ± SD.

Results

MUTATION OF *HMGCRB* INDUCES CRANIOFACIAL DEFECTS IN ZEBRAFISH

To investigate a possible role of HMGCR in orofacial morphogenesis, we used the *hmgrcb*$^{s617/s217}$ line in which failure in heart-tube morphogenesis and pericardial edema were previously described (D'Amico et al., 2007). We found that *hmgrcb* mutation produced an important reduction of head size and shortening of the anterior–posterior axis (data not shown) with severe defects of craniofacial development (Fig. 1).

In WT embryos at 4.5 dpf, Alcian blue staining revealed a normal craniofacial cartilaginous skeleton (Fig. 1A–C). Heterozygous siblings had either normal phenotype (26%, Table 1, which is available online) or a mild cleft with an intermediate shortening of anterior neurocranium (ANC), which is analogous to the amniotes palate (compare Fig. 1E with 1B; 46%, moderate phenotype in Table 1). Slight reduction in the jaw and gill apparatus length was also observed (Fig. 1D, F). In affected-sibling,

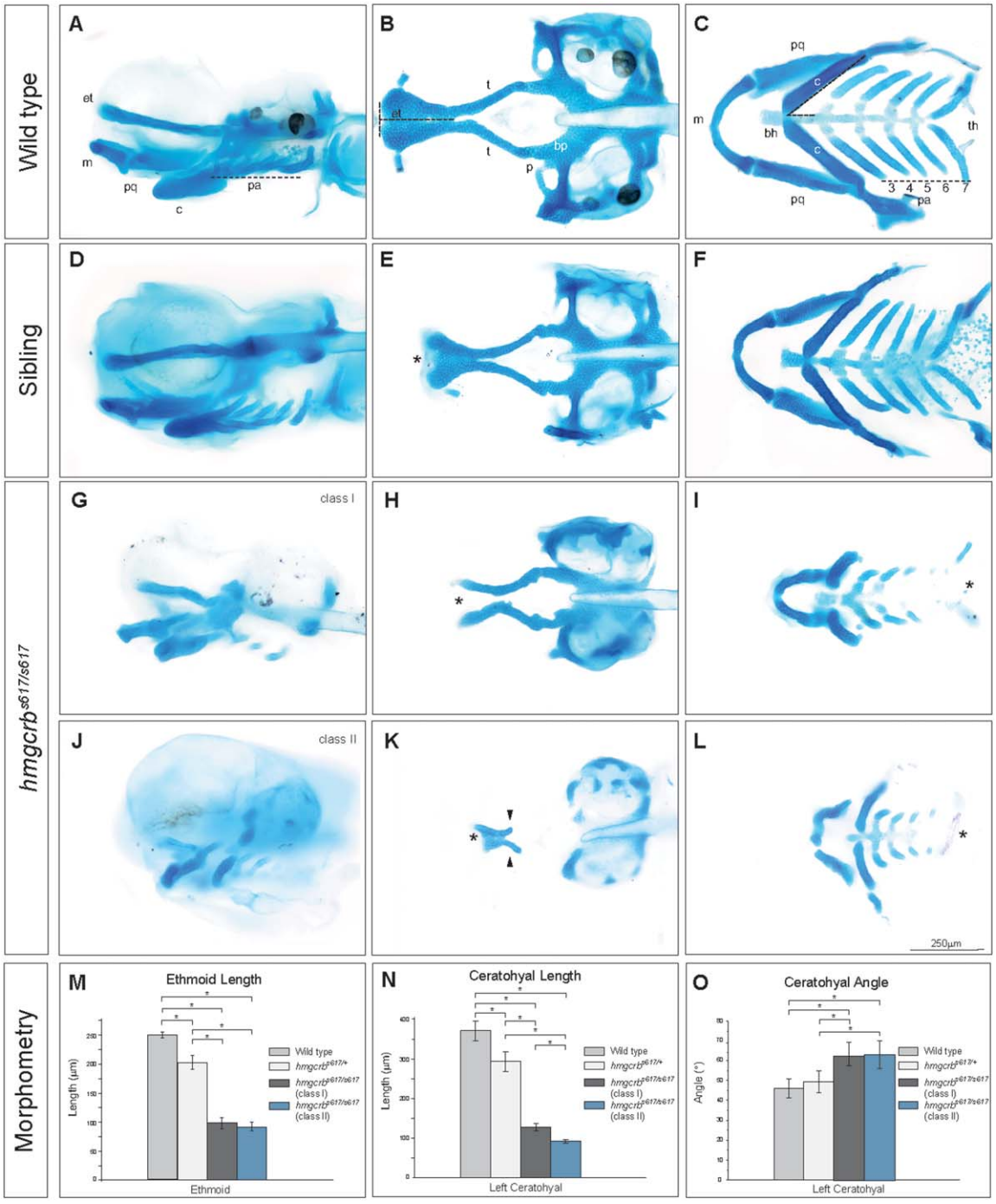

FIGURE 1. *Mutation of* **hmgcrb** *induces craniofacial defects in cartilaginous structures that include palatal clefts in zebrafish. Views of flat-mounted Alcian blue staining of orofacial structures of zebrafish at 4,5 dpf: lateral (A,D,G,J); dorsal (neurocranium) (B,E,H,K); ventral (viscerocranium) (C,F,I,L). Anterior to the left.* **A–C**: *Normal cartilaginous elements of cartilaginous craniofacial structures composed by maxillary elements (ethmoid and trabeculae), mandible (Meckel's cartilage and palatoquadrate, hyoid arch, ceratohyal, and hyosymplectic), and five posterior branchial arches (3–7) (ceratobranchial cartilage).* **D–F**: *Moderate shortening in trabeculae and ethmoid plate with mild cleft (**asterisk** in E) in affected-sibling embryos. Mandible elements presented slight reductions of length (F).* **G–L**: *Class I mutants displayed severe phenotypes with short orofacial elements and cleft (**asterisk** in H); Class II mutants displayed reduction of cartilage staining with extensive shortening of anterior ethmoid plaque (**asterisk** in K) and viscerocranium structures. Absence of polar cartilage and basal plaque only with the presence of distal trabeculae in neurocranium is observed (**arrowheads** in K). Enlargement of the angle between ceratohyal and the midline (C, **dotted line**) is observed in both class of mutants (I,L).* **M–O**: *Graphs show the reduction in the ethmoid plate and certohyal length in mutant and affected-sibling in comparison to WT embryos (M,N; $p < 0.05$, $n = 10$ per condition). The angle of both right and left ceratohyal cartilages increases in HMGCR loss-of-function, only the left is shown because both have similar values (O; $p < 0.05$; $n = 9$ per condition).* **Asterisks** *indicate statistical significance. Error bars: mean ± SD. et, ethmoid; t, trabecula; Meckel's cartilage; pq, palatoquadrate; c, ceratohyal; pa, pharyngeal arches; p, polar cartilage; bp, basal plaque; bh, basihyal; th, teeth. Scale bar = 250 µm.*

structures of the ANC were narrower than in WT showing a significant reduction in length of the lateral ethmoid plate together with a little loss of tissue in the more distal medial ethmoid and defects in fusion between median and lateral ethmoid, resulting in mild cleft (asterisk in Fig. 1E).

In *hmgcrb* mutant embryos, we recognized two classes of severe phenotypes: (i) Class I mutants presented severe reduction of length in the lateral ethmoid and absence of the medial ethmoid, thereby creating a palatal cleft (asterisk in Fig. 1H) plus a dimorphic basal plaque. Viscerocranium elements were short, narrow, and had less Alcian blue staining (Fig. 1G–I, M; 4%, severe phenotype in Table 1); (ii) Class II mutants (24%, severe phenotype in Table 1; Fig. 1J–M) showed an extensive shortening of the ethmoid plate (asterisk in Fig. 1K) and distal trabeculae were partially formed (arrowhead in Fig. 1K), while proximal trabeculae, polar cartilage, and basal plaque were absent (compare Fig. 1K with 1B).

Analysis of the lower jaw skeleton revealed reduced and dysmorphic ceratohyal, Meckel's cartilage, palatoquadrate, and pterygoid process of the palatoquadrate, with weak Alcian blue staining in posterior pharyngeal arches and enlargement of the angle between ceratohyal and the midline (Fig. 1L). The right and left ceratohyal were deflected caudally and short when compared with the control (compare Fig. 1I and L with 1C). Loss of chondrocytes and no-intercalated pattern of chondrocytes were observed using 40 × magnification in palate, excluding insufficient Alcian blue staining (data not shown). In addition, the earliest-formed teeth were lost in the two classes of mutants (asterisk in Fig. 1F, I, L). Morphometric analysis of orofacial structures showed a significant decrease in the size of cartilaginous elements in mutants and affected-sibling compared with WT embryos (Fig. 1M, N). These results suggest that abrogation of *hmgcrb* induced orofacial malformation including OFCs.

TEMPORALLY RESTRICTED PHARMACOLOGICAL INHIBITION OF HMGCR REVEALS A DIFFERENTIAL REQUIREMENT FOR HMCGR DURING EARLY AND LATE PHASES OF OROFACIAL DEVELOPMENT

Mutation of *hmgcrb* did not reveal the time at which HMCGR specifically modulates orofacial morphogenesis. Thus, we took advantage of the zebrafish model, and we performed a temporally restricted disruption of HMGCR activity using atorvastatin during (i) NCCs migration toward pharyngeal arches and subsequent condensation (10–24 hpf, early inhibition assay) and (ii) during chondrogenesis (30–48 hpf, late inhibition assay).

In early-treated larvae, HMCGR inhibition by statin produced slightly small heads (data not shown), mildly reduced craniofacial skeletal elements (compare Fig. 2A–C with 2D–F; 96%, Table 1) and a minor OFC (asterisk in Fig. 2E), as revealed by cartilage and bone staining at 4.5 dpf. Reduction of the ceratohyal angle (Fig. 2I, N) was also observed. Morphometric analysis showed that the decrease in craniofacial structures length in early assay is not statistically significant with the only exception of the change in ceratohyal angle (Fig. 2M–P).

On the other hand, late inhibition of the HMGCR enzyme induced significant defects in craniofacial development and predominantly abnormal formation of ANC in a dose-dependent manner (2 μM, 3 μM, 4 μM, 5 μM of atorvastatin) (Table 2). Higher doses of atorvastatin were toxic and induced high rates of mortality (data not shown).

In particular, late-treated embryos displayed severe shortening of ethmoid plate and trabeculae, polar cartilage and basal plaque were almost absent, and the Alcian blue staining was significantly diminished (compare Fig. 2H with 2K; 95%, Table 1). Also, prospective mandible presented shortening of palatoquadrate, pterygoid process of the palatoquadrate, ceratohyal, and Meckel's cartilages, and less Alcian blue staining in pharyngeal arches (compare Fig. 2I and L with 2C). The ceratohyal angle was increased, as shown in Figure 2L, R. Similar to that found in mutant embryos, statin late-treated embryos displayed disruption of normal pattern of chondrocytes intercalation in cartilages elements (data not shown). Furthermore, only late statin-treated embryos showed an absence of paresphenoid bone and earliest-formed teeth (asterisks in Fig. 2K, L). Morphometric analysis revealed that ethmoid, trabeculae, and ceratohyal cartilages of late-treated embryos are statistically different from the WT (Fig. 2M–T).

Next, we investigated the possibility that orofacial malformations might be a consequence of defects induced during earlier stages of development. We also performed incubation with atorvastatin during gastrulation (4–8 hpf), which resulted only in minor defects in cartilages formation (data not shown). This result is in agreement with previous studies and with the observation that in *hmgcrb* mutant pharyngeal endoderm and early morphogenesis are unaffected (Thorpe et al., 2004; Eisa-Beygi et al., 2013).

Our data suggest that HMCGR loss of function disrupted the normal craniofacial development resulting in different orofacial deformations, including palatal cleft, that varied in severity depending on the level of HMGCR inhibition and on the moment in which HMGCR is disrupted.

Disruption of HMGCR Function Primarily Affects Postmigratory CNCCs

In zebrafish, as in other vertebrates, pharyngeal cartilages originate from NCCs (review in Szabo-Rogers et al., 2010). Thus, we desired to determine which events of orofacial development might be specifically affected by the lack of HMGCR. We evaluated the effect of the inhibition of this enzyme on the migration toward pharyngeal arches and the subsequent condensation of the CNCCs.

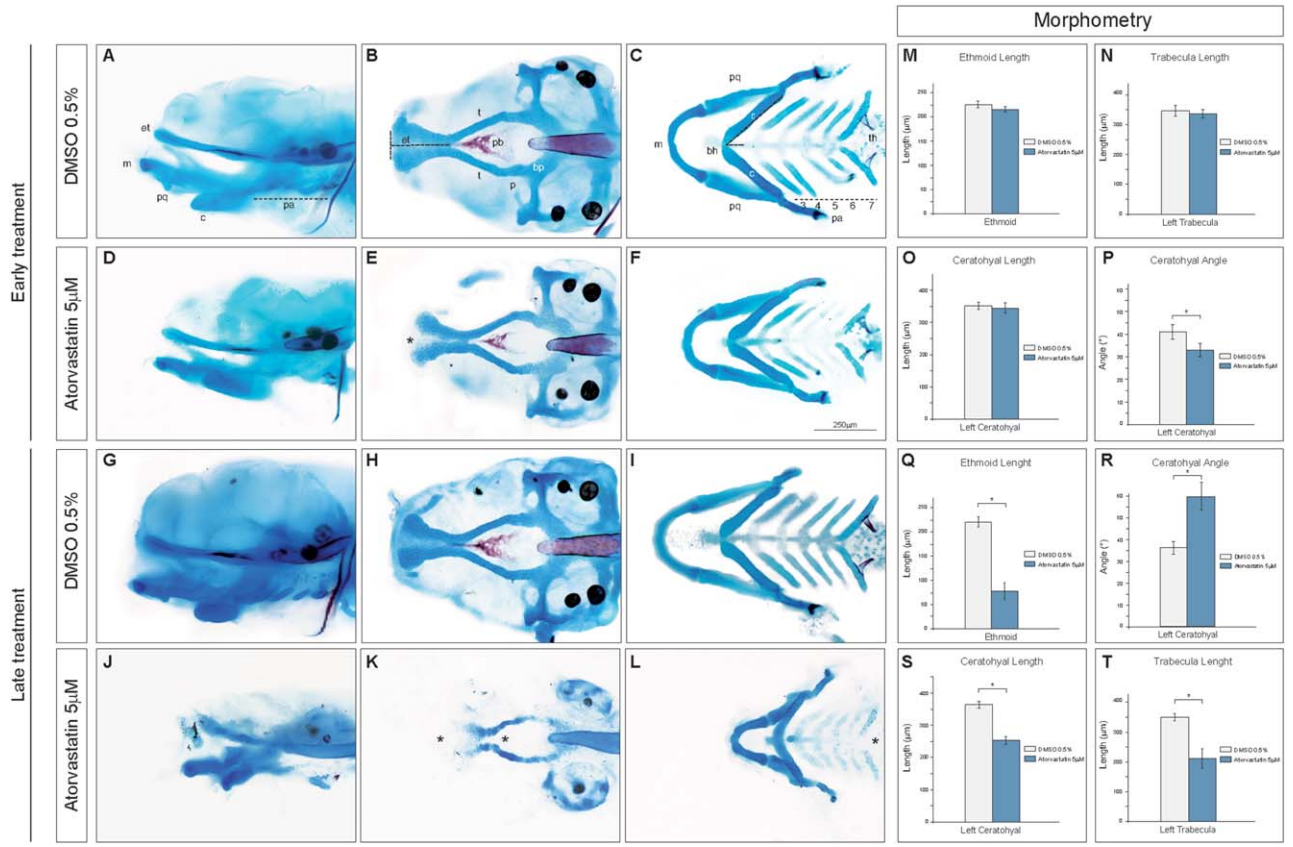

FIGURE 2. *Early and late statin-treatment differentially disrupts orofacial morphogenesis in zebrafish. A, D, G, and J lateral; B, E, H, and K dorsal; C, F, I, and L ventral views of flat-mounted cartilage and bone stained of zebrafish larvae at 4.5 dpf; anterior to the left.* **A–C,G–I***: Normal cartilaginous orofacial structures are observed in DMSO 0.5% treated embryos during early and late stage of craniofacial development.* **D–F***: Atorvastatin-treated embryos during early phase of orofacial morphogenesis show a weak effect on the neurocranium, specifically in ethmoid plate with a slight shortening and milder clefts (E,* **asterisk***). The viscerocranium presents a normal pattern, with a reduction of angle between ceratohyal and midline (F).* **J–L***: Pharmacological treatment with statin during chondrogenesis produced severe shortening of ANC (palate) and viscerocranium cartilage elements, reduction of Alcian blue staining in neurocraniom and pharyngeal arches, loss of polar cartilage, basal plaque, paresphenoid bone, and teeth primordia (K and L).* **M–S***: Quantification of craniofacial structures length (M–O and P–S, $p < 0.05$; $n = 10$ per condition) and measurement of left ceratohyal angle (N and Q, $p < 0.05$; $n = 10$ per condition, both left and right ceratohyal angle were equivalent).* **Asterisks** *indicate statistical significance. Error bars: mean ± SD. et, ethmoid; t, trabecula; Meckel's cartilage; pq, palatoquadrate; c, ceratohyal; pa, pharyngeal arches; p, polar cartilage; bp, basal plaque; bh, basihyal; pb, paresphenoid; th, teeth. Scale bar = 250 µm.*

First, we performed an atorvastatin early inhibition assay (10–24 hpf) in the *Tg(-4.9sox10::GFP)*^{ba2} line to abrogate HMCGR during CNCCs migration toward the facial primordia. At 24 hpf, a slight reduction of GFP-positive CNCCs in all pharyngeal arches of treated embryos was observed, especially in the mandible arch (pa1), the stomodeum, ethmoid, and trabeculae precursors (compare Supp. Fig. S1A, B with Supp. Fig. S1C, D). At the same time, trunk NCCs (TNCCs) of atorvastatin-treated embryos showed slightly delayed migratory streams with a normal pattern (Supp. Fig. S1A, C). These results are concordant with the mild craniofacial phenotypes observed in Alcian blue stained embryos exposed to the same treatment and stage of development (Fig. 2D–F).

Interestingly, dorsal views revealed that GFP-positive cells remained separated at the midline, supporting that the phenotype observed might not be due to fusion in the absence of medial structures (Supp. Fig. S1D). This result differs from the one observed in zebrafish injected with morpholinos targeting zebrafish *shh* in which fusion at midline is indeed observed (Wada et al., 2005).

In addition, we analyzed by whole-mount *in situ* hybridization the expression of genetic markers to investigate possible defects in NCCs specification, migration, condensation, and survival. We tested the expression of *sox10*, as a genetic marker for specification and migration of NCCs (Carney et al., 2006). In premigratory CNCCs, at 14 hpf, *sox10* signal in affected-sibling and *hmgcrb* mutants appeared to be mildly reduced. Nevertheless their

migration was delayed, as can be noticed taking the posterior edge of the optic vesicle as a reference. TNCCs migration was also delayed in affected-sibling and mutants at 14 hpf (Fig. 3B, C).

At 24 hpf, affected-sibling and *hmgcrb* mutants showed mild and moderate reduction of *sox10* levels in postmigratory CNCCs, respectively (Fig. 3E, F, compare with 3D). In addition, at the same stage, *sox10* expression was reduced in CNCCs in atorvastatin-treated embryos similar to the affected-siblings (Supp. Fig. S1B; Fig. 3E). Also, TNCCs of mutants and affected-siblings displayed a modest delay in migration (Fig. 3E, F). The arch marker *dlx2a* is a gene involved in condensation and survival of CNCCs (Sperber et al., 2008). A significant reduction of *dlx2a* expression was detected in mutants within the four first pharyngeal arches at 24 hpf (Fig. 3I; 25%, Table 3). Affected-sibling and early atorvastatin-treated embryos also displayed moderate reduction of *dlx2a* expression in all pharyngeal arches (Fig. 3H; Supp. Fig. S2D; 52% and 100%, Tables 3, 4).

These results suggest that HMGCR disruption had an impact predominantly on CNCC condensation and/or survival/proliferation and to a lesser extent on CNCC migration according to the grade of HMGCR inhibition and the stage of orofacial development in which it occurs.

HMGCR LOSS-OF-FUNCTION DISRUPTS CHONDROGENESIS OF OROFACIAL STRUCTURES IN ZEBRAFISH

To study whether loss of HMGCR activity plays a role in chondrogenesis, we analyzed the expression of chondrogenic markers. We found that expression of *shha*, a gene required for patterning and differentiation of cartilage and bone (Xavier et al., 2016), displayed no significant differences between affected-sibling, *hmgcrb* mutants, early and late atorvastatin-treated, and control embryos at 48 hpf (Fig. 3J–L; Fig. 4A–C; Supp. Fig. S2E–G). However, we detected different grades of shortening in its expression domain in the orofacial ectoderm and pharyngeal endoderm under the different experimental conditions (arrowheads in Fig. 3K,L, Fig. 4C, and Fig. 2G; Tables 3, 4, 5).

gli1 is an effector of SHh signaling and is required for orofacial development in zebrafish (Schwend et al., 2010). Hh signaling secreted from ventral brain primordium at the end of gastrulation established the stomodeum, and this structure interacts with postmigratory CNCCs to generate anterior craniofacial skeleton (Eberhart et al., 2006). Then, the SHh signal emanating from orofacial ectoderm, ventral brain, and pharyngeal endoderm is fundamental for chondrogenesis and outgrow of ANC and pharyngeal arches (Wada et al., 2005; Schwen and Ahlgren, 2009). Our results showed that *shha* and *gli1* were normal at 10 hpf in *hmgcr*b mutants (Supp. Figs. S3B, S3D; 100%, *shha:* $n = 132$ and *gli1:* $n = 142$).

Of interest, we found that *gli1* at 52 hpf presented a small reduction and faint expression of its transcript in the orofacial ectoderm and in ventral brain of affected-sibling and *hmgcrb* mutants fish, respectively (arrowhead and asterisk in Fig. 3, O; 51% and 28%, Table 3). In pharyngeal endoderm, affected-sibling and mutants embryos showed, respectively, mild and severe decreased levels of *gli1* together with shortening in its expression domain (arrowhead in Fig. 3, O). Late inhibition of HMGCR produced levels of *gli1* expression that are intermediate between those detected in affected-sibling and mutants (compare Fig. 4E, F with Fig. 3O; 85%, Table 5). Despite of this, expression of the *gli1* gene in affected-sibling and embryos with early and late atorvastatin treatment was retained at normal levels in others structures such as pectoral fins, floor plate, among others structure where *gli1* is expressed, whereas mutant fish presented a moderate reduction (data not shown). In contrast, early-treated embryos showed normal *gli1* expression in craniofacial structures with mild shortening of its domain expression (arrowhead in Supp. Fig. S3J).

At 54 hpf, we found that expression of the gene encoding type II collagen (*col2a1*), essential for chondrogenesis (Yan et al., 1995) in notochord and the epithelium of the otic capsule, was unchanged in controls, mutant, affected-sibling, and atorvastatin-incubated larvae (data no shown). In the ANC, however, heterozygous affected-siblings displayed loss-of-expression in the most anterior ethmoid plate region, although trabecular condensations and more posterior ethmoid plate had normal levels of *col2a1* (asterisks in Fig. 3Q; 50%, Table 3). In mutants, *col2a1* was either absent or reduced in the medial ethmoid and more anterior region of lateral ethmoid (asterisks in Fig. 3R, S; 23% and 3%, Table S3). These results are concordant, respectively, with class II and class I phenotypes observed in cartilage staining (compare Fig. 3R, S with Fig. 1H, 1K). Consistent with this, in late-treated embryos, *col2a1* expression during chondrogenesis was completely absent in the ethmoid plate, whereas more posterior trabecular cartilages presented expression of this transcript (asterisk in Fig. 4F; 100%, Table 5). Early atorvastatin-treated embryos displayed a milder phenotype (Supp. Fig. S2L; Table 4). These findings suggest that HMGCR inhibition disrupted chondrogenesis probably due to a failure in SHh signaling without discarding a role for other signaling pathways.

Discussion

This report presents the first evidence that HMGCR inhibition in zebrafish induced a spectrum of orofacial anomalies that included OFCs, probably due to reduction in cholesterol and intermediates metabolites of MVA pathway. Severity of craniofacial malformation varied depending on the grade of HMGCR inhibition and according to the developmental stage in which HMGCR function was inhibited. A similar situation is observed in syndromic OFCs induced

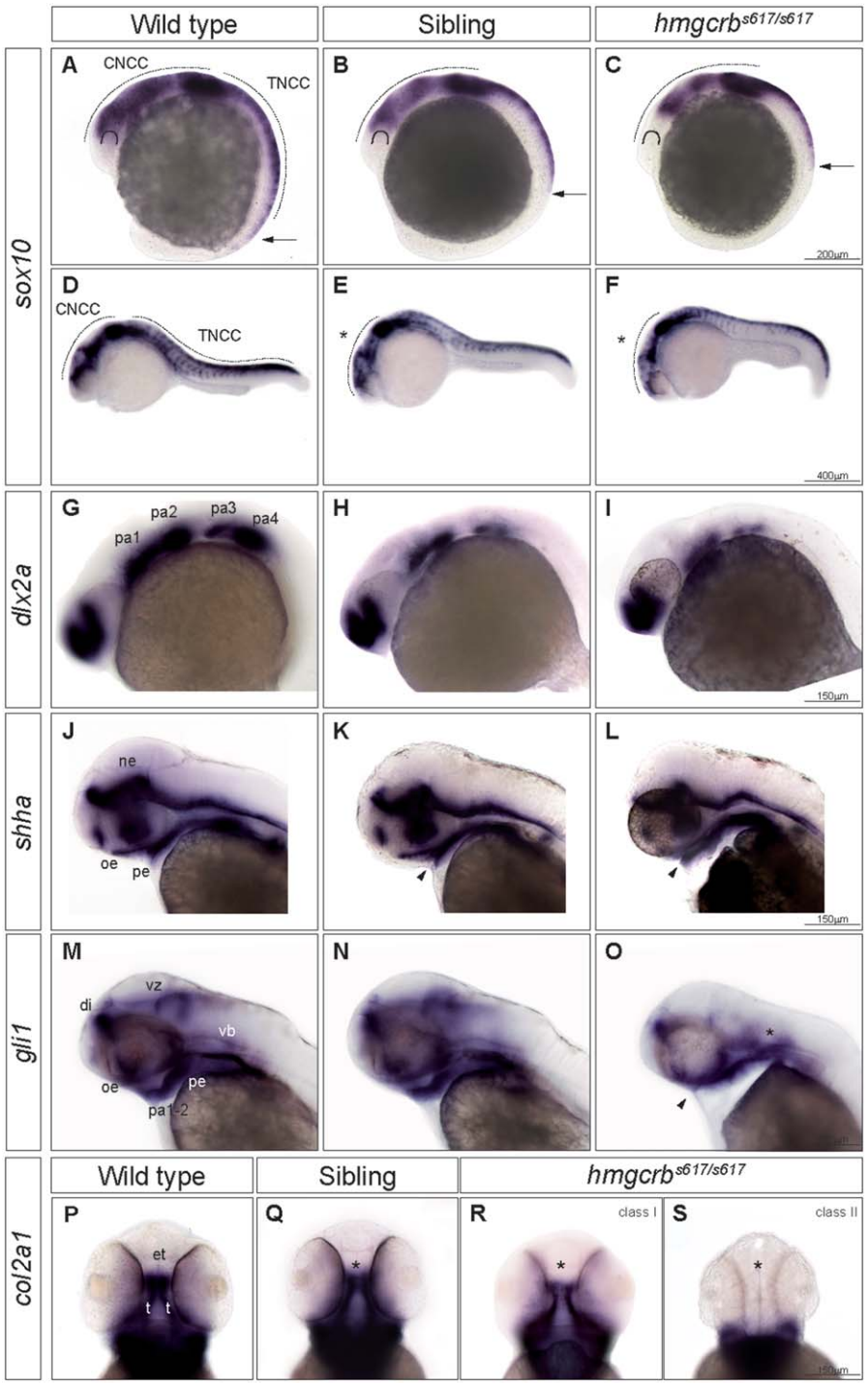

FIGURE 3. *Loss of HMGCR function affects primarily postmigratory CNCCs and chondrogenesis. A–O: Lateral; P–S, ventral views of WT, affected-sibling, and* ***hmgcrb*** *mutants embryos processed by whole-mount in situ hybridization.* **A–C**: *Decreased* ***sox10*** *expression in affected-sibling and mutants (**asterisks** in B,C). Delay in migration of more anterior premigratory CNCCs (**lines** delineate the posterior edge of the optic vesicle in A–C) and TNCCs at 14 hpf (10 somites) in mutant and affected-sibling (**arrows** in B,C).* **D–F**: *Loss of HMGCR function leads to discrete and moderate reduction in* ***sox10*** *expression in frontonasal CNCCs at 24 hpf in siblings and mutants (**asterisks** in E,F). Expression in TNCCs displayed a mild reduction in both conditions (E,F).* **G–I**: *At 24 hpf, **dlx2a** expression is diminished in pharyngeal arches of affected-sibling (H) and mutants (I).* **J–L**: *At 48 hpf, **shha** is expressed in neurocranium, oral ectoderm, pharyngeal endoderm, and pharyngeal ectodermal margin in WT embryos (J). Affected-siblings and mutants show a slightly shortened expression domain in oral ectoderm with normal levels of **shh** transcript (**arrowheads** in K,L).* **M–O**: *Reduction of **gli1** expression at 52 hpf is observed in ventral brain, oral ectoderm, and pharyngeal endoderm in affected-sibling. **hmgcrb** mutants embryos present almost absence of **gli1** expression in ventral brain (**asterisk** in O) and moderate reduction of this gene in branchial arches and in oral ectoderm (**arrowhead** in O) compared with WT (M). Affected-sibling and mutants show shortening of **gli1** domain expression in lower and upper jaw (**arrowhead** in N, O).* **P–S**: ***col2a1*** *expression is normal in trabeculae with moderate shortening of its expression domain in the ethmoid of affected-sibling at 54 hpf (**asterisk** in Q). The expression of **col2a1** is reduced (**asterisk** in R) or totally absent (**asterisk** in S) in ANC elements of mutants, but most posterior neurocranium has normal levels. CNCCs, cranial neural crest cells; TNCCs, trunk neural crest cells; et, ethmoid plate; t, trabecule; pa, pharyngeal arches; m, Meckel's cartilage; oe, oral ectoderm; pe, pharyngeal endoderm; ne, neuroectoderm; di, diencephalon; vb, ventral brain; vz, ventricular zone. Scales bars = 200 µm in A–C; 400 µm in D–F; 150 µm in G–S.*

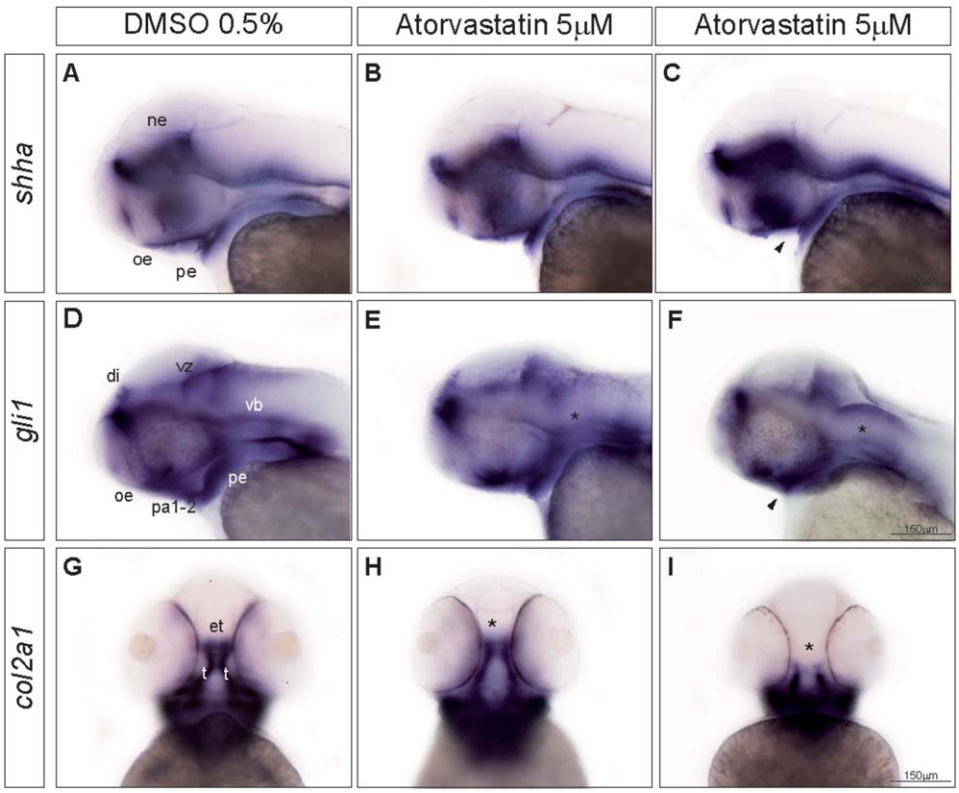

FIGURE 4. *Pharmacological treatment with atorvastatin during late stage of orofacial morphogenesis induces variable defects in chondrogenesis gene expression. A–F: Lateral; G,H, ventral views of DMSO 0.5% and statin late-treated embryos labeled with chondrogenic RNA probes by whole-mount in situ hybridization.* **A–C***: At 48 hpf,* ***shha*** *is expressed normally in neurocranium, oral ectoderm, pharyngeal endoderm, and pharyngeal ectodermal margin in embryos incubated with atorvastatin during late stage of orofacial formation (B), while a small group of embryos show a slightly shortened domain of* ***shh*** *expression in oral ectoderm (****arrowhead*** *in C).* **D–F***: Important reductions of* ***gli1*** *expression in ventral brain (****asterisks*** *in E,F), oral ectoderm, and pharyngeal elements of late-treated embryos at 52 hpf. A small group of these embryos display shortened* ***gli1*** *expression in oral ectoderm and pharyngeal endoderm domain (****arrowhead*** *in F).* **G–I***: Two phenotypes are observed for* ***col2a1*** *expression at 54 hpf. Loss of* ***col2a*** *transcripts in more anterior ethmoid region is observed in a minor group of embryos (****asterisk*** *in H). The expression of* ***col2a1*** *is absent in the ethmoid plate domain and is observed only in most posterior region of trabeculae (****asterisk*** *in I). The expression in the most posterior neurocranium is similar to DMSO control (G). et, ethmoid plate; t, trabecule; pa, pharyngeal arches; m, Meckel's cartilage; oe, oral ectoderm; pe, (pharyngeal endoderm; ne, (neuroectoderm; di, (diencephalon; vb, ventral brain; vz, (ventricular zone). Scale bar = 150 μm.*

by disorders in postsqualene cholesterol pathway in human and animal model (reviewed in Moebius et al., 2000; Porter and Herman, 2011).

Our study revealed that the most severe phenotype was the extensive loss of the ANC with distinct sensibility of ANC and viscerocranium displayed by *hmgcrb* class II mutants, followed by atorvastatin late-treated embryos with a less severe phenotype. In contrast, in early-treated embryos, the orofacial phenotype was milder. Affected-sibling embryos presented an intermediated shortening of craniofacial structures phenotype without loss of cartilage elements that suggests a possible correlation between residual HMGCR activity and the severity of induced orofacial malformations. Consistent with this, atorvastatin induced variable craniofacial malformations in a dose-dependent manner, suggesting a differential level of the HMGCR activity when embryos are incubated in late stages of craniofacial morphogenesis. This result supports the idea that the spectrum of craniofacial phenotypes may be due to differential activity of the HMGCR in the experimental conditions of this study.

In addition, defects in structures of the orofacial midline or failure in extension of palate (ethmoid plaque) were observed with different degree of severity in early statin-treated embryos, affected-sibling, and in a small group of mutants (Class I). Midline malformations can be interpreted in zebrafish as diverse grade of OFC because shortened trabecular cartilages (maxillary prominences in amniotes) and/or loss of medial ethmoid (frontonasal prominence in amniotes) represent clefts in this model according to Swartz et al. (2011) and Dougherty et al. (2013). Analogous defects are observed in the mouse

TABLE 1. *Craniofacial Malformation due to Inhibition of HMGCR Function*

		hmgcrb^{s617} line			Early statin-treated		Late statin-treated	
Phenotype	**WT**	**Normal sibling**	**Affected-sibling**	**Mutants**	**DMSO 0.5%**	**5 μM**	**DMSO 0.5%**	**5 μM**
Normal	387 (100%)	90 (27%)	-	-	247 (100%)	-	192 (100%)	-
Mild								
Small shortening of orofacial structures and milder OFCs	-	-	-	-	-	251 (96%)	-	4 (2%)
Moderate								
Intermediate shortening of orofacial structures with milder OFCs	-	-	145 (43%)	-	-	8 (3%)	-	6 (3%)
Severe: Class I								
Extensive shortening of orofacial structures, partial loss of cartilage structure and OFCs	-	-	-	11 (3%)	-	3 (1%)	-	-
Severe: Class II								
Extensive shortening of orofacial structures and loss of structures of anterior neurocranium	-	-	-	85 (27%)	-	-	-	179 (95%)
Total (n)	**387**		**304**		**247**	**262**	**192**	**189**

Craniofacial phenotype categories: Mild (ceratohyal length: 300–330 μm; ethmoid: 380–$\leq$440 μm); Moderate (ceratohyal length: 250–$\leq$300 μm; ethmoid: 200–$\leq$380 μm); Severe (ceratohyal length: $<$ 250 μm; ethmoid: $<$ 200 μm).

when cleft of lip and palate is a consequence of failure in extension of the palatal shelves that restrain midline union (Ito et al., 2003).

Dissimilar phenotypes were observed in early and late inhibition of HMCGR, revealing a differential requirement for HMCGR during different phases of orofacial development. When HMGCR inhibition occurred during early stage of craniofacial morphogenesis (10–24 hpf), we found that CNCCs migration and mediolateral polarity (midline tissue expansion) were barely affected in the transgenic line *Tg(-4.9sox10::GFP)*^{ba2}. Statin treatment between 30 and 48 hpf disrupted predominantly PA1 chondrogenesis but did not affect CNCC morphogenesis at the midline. Our results indicate that the cases of palatal cleft displayed by Class I mutants were likely caused by impaired ANC chondrogenesis with normal CNCC separation at the midline, suggesting that early phase of craniofacial morphogenesis was slightly affected.

The same process might be also occurring in Class II mutants, but severe shortening and massive loss of cartilage could be hiding this phenomenon. According to this, the expression of *sox10* signal in premigratory and postmigratory CNCCs was only mildly reduced in affected-sibling, in early and late-treated embryos. Also, decreased expression of *dlx2a* in pharyngeal arches, *col2a1* in ANC and *gli1* in oral ectoderm, pharyngeal endoderm, and ventral brain of mutants and in late-treated embryos supported a role of HMGCR predominantly after CNCCs migration to pharyngeal arches, possibly modulating CNCCs condensation and chondrogenesis phases during orofacial development.

The SHh pathway is an important signaling pathway involved in craniofacial development, and its alterations mediated by impairment in the cholesterol pathway have been proposed as the cause of many neurodevelopmental, skeletal, and craniofacial development defects (Bale, 2002; Porter and Herman, 2011; Xavier et al., 2016). However, this idea arises principally from considering the known role of Hh signaling as a morphogen during development of these tissues together with the fact that Hh signaling is perturbed by defects in cholesterol metabolism (Stottmann et al., 2011). Although the connection may seem almost

TABLE 2. *Dose-Dependent Effects on Craniofacial Malformations Induced by Atorvastatin Treatment during Late Stage of Orofacial Morphogenesis in Zebrafish*

	Craniofacial phenotype categories				
Pharmacological treatment	**Normal**	**Mild**	**Moderate**	**Severe**	**Total (n)**
DMSO 0.5%	176 (100%)	-	-	-	176
Atorvastatin					
2 μM	15 (7.7%)	172 (88.7%)	7 (3.6%)	-	194
3 μM	-	107 (59.8%)	69 (38.5%)	3 (1.7%)	179
4 μM	-	19 (10.5%)	140 (77.3%)	22 (12.2%)	181
5 μM	-	-	35 (18.8%)	151 (81.2%)	186

obvious, the mechanistic relation between these events needs further investigation. Our study provided direct evidence supporting this idea, and it showed that late orofacial development is particularly sensitive to the loss of HMGCR function and its impact on SHh signaling occurring during late phases.

We showed that *shha* expression levels were normal in all the tested experimental conditions. However, variability in reduced expression of its effector *gli1* was observed at 52 hpf in ventral brain, oral ectoderm, and pharyngeal endoderm of mutants, atorvastatin-incubated embryos during chondrogenesis, and affected-sibling. These results show that the activation of SHh signaling is defective due to perturbation in the MVA pathway. In addition, supporting the late HMCGR requirement for SHh signaling activation, early inhibition assay led to normal levels *of gli1* transcript.

To observe cartilage morphogenesis defects in HMGCR loss-of-function, we tested specific expression of early chondrogenic markers *col2a1* (Yan et al., 1995). In mutant and late statin-treated embryos, anterior neurocranial expression was completely absent, while posterior neurocranial expression remains. In contrast, the effect in early-treated embryos was minimum. These findings are consistent with a failure predominantly in chondrogenesis of the facial primordia as it was observed in *smo*$^{-}$ and *con/disp1* mutants (*disp1* gene is involved in dispersion of Hh ligands into the extracellular field) (Eberhart et al., 2006; Schwend and Ahlgren, 2009). Furthermore, incubation with CyA (an antagonist of Hh signaling) during late stages of craniofacial morphogenesis disrupts ANC and pharyngeal chondrogenesis without producing NCC morphogenesis defects at the midline (Wada et al., 2005; Eberhart et al., 2006; Schwend and Ahlgren, 2009). We found a similar phenotype in late-treated embryos. Finally, only *hmgcrb* mutants and late-treated embryos showed absence of mature teeth, whereas in sibling and early-treated embryos teeth development was normal, consistently with requirement of *shha* during late stage of odontogenesis (Jackman et al., 2010).

However, the high degree of variability observed in phenotypes resulting from an impairment of cholesterol metabolism cannot be overlooked and suggests a more complicated scenario. For example, an important portion of HMGCR mutants (Class II) displayed loss of several cartilaginous elements predominantly in ANC, but this loss was not so severe as the phenotype observed in the Hh-receptor *Smoothened* (*Smo*) mutation, which can lead to an almost complete elimination of maxillary craniofacial skeletal elements in zebrafish and mouse (Chiang et al., 1996; Chen et al., 2002; Eberhart et al., 2006). Furthermore, hypomorphic mutants of Hh in fish presented trabecular fusion and absence of medial ethmoid (Wada et al., 2005), but the fusion phenotype was not observed in any of the experimental conditions analyzed in this study.

Indeed, Class I mutants presented short lateral ethmoid plaques and absence of medial ethmoid without midfacial fusion. CyA incubation of zebrafish embryos during gastrulation (4–8 hpf) caused midfacial fusion (Wada et al., 2005). However, in the current study incubation with atorvastatin in the same period produced only minor ANC defects (data not shown), similar to that found in embryos incubated with atorvastatin during CNCC migration to facial primordia. Our results suggested that mediolateral polarity (midline tissue expansion) during the early stage of craniofacial morphogenesis was barely affected in HMGCR inhibition, consistent with normal *gli1* expression during early stage in ventral brain primordium in *hmgcrb* mutants. Thus, cartilage loss, shortening in orofacial structures, and in some cases, palatal cleft in *hmgcrb* mutants is likely to be produced by defects in postmigratory CNCCs during condensation and chondrogenesis phases.

Notably, reduced expression of *dlx2a* and *sox10* in postmigratory CNCCs was observed mainly in mutants, suggesting failure in condensation or survival/proliferation of postmigratory CNCCs. It has been shown that loss of Hh signaling has a negative impact on the ability of CNNCs to condense in the oral stomodeum (Eberhart et al., 2006). Interestingly, SHh mediates cell survival in PA1 blocking *cdon* pro-apoptotic activity in zebrafish (Delloye-Bourgeois

TABLE 3. *Expression of Craniofacial Genetic Markers in WT, Sibling, and hmgcrb$^{s617/s617}$ Embryos*

Genotype / phenotype	WT	Sibling (*hmgcrb*$^{+/+}$ *or hmgcrb*$^{s617/+}$)	*hmgcrb*$^{s617/s617}$
sox10 (14 hpf)			
Normal	57 (100%)	15 (27%)	-
Mild reduction of expression in pre-migratory CNCCs and delay in migration with normal mRNA levels in otic placode	-	27 (48%)	14 (25%)
sox10 (24 hpf)			
Normal	45 (100%)	24 (25%)	-
Mild reduction of expression in CNCCs with normal mRNA levels in otic placode	-	51 (54%)	-
Moderate reduction of expression in CNCCs with normal mRNA levels in otic placode	-	-	21 (22%)
dlx2a (24 hpf)			
Normal	250 (100%)	76 (23%)	-
Moderate reduction of expression in pharyngeal arches	-	172 (52%)	-
Severe reduction of expression in pharyngeal arches	-	-	84 (25%)
shha (48 hpf)			
Normal	80 (100%)	69 (22%)	-
Mild reduction of domain extension in oral ectoderm		168 (53%)	
Moderate shortening in domain extension in oral ectoderm and pharyngeal endoderm	-	-	80 (25%)
gli1 (52 hpf)			
Normal	112 (100%)	34 (21%)	-
Small reduction in ventral brain and mild decrease of expression in oral structures with shortening in its domain of expression	-	83 (51%)	-
Loss of expression in ventral brain and oral ectoderm and severely decreased expression and shortening in pharyngeal endoderm domain	-	-	46 (28%)
col2a1 (54 hpf)			
Normal	365 (100%)	102 (24%)	-

TABLE 3. *Continued*

Genotype / phenotype	WT	Sibling (*hmgcrb*$^{+/+}$ *or hmgcrb*$^{s617/+}$)	*hmgcrb*$^{s617/s617}$
Shortening of domain expression in the most anterior region of ethmoid plaque	-	204 (50%)	-
Shortening of domain expression in lateral ethmoid and loss of expression in medial ethmoid	-	-	11 3%
Absence of expression in ethmoid plate and trabeculae domains	-	-	94 (23%)

et al., 2014), and SHh loss-of-function in oral ectoderm and pharyngeal endoderm increased cell death in postmigratory NCCs and affected cartilage condensation of PA1 (Brito et al., 2006; Billmyre and Klingensmith, 2015). In addition, statins have a pro-apoptotic effect in different in vitro (normal and cancer cells) and in vivo models mediated by the reduction in Bcl-2 protein levels and autophagy induction (Wood et al., 2013; Dykstra et al., 2015). Also, statins may inhibit the cell cycle in cancer cells (Matusewicz et al., 2015). Diminished survival of postmigratory CNCCs may also be consequence of these statin effects. These conclusions require further investigation to be confirmed.

Importantly, the early SHh signal emanating from the ventral brain regulates the stomodeum establishment that subsequently promotes, in an Hh-signaling independent manner, the CNCCs condensation that will result in ANC and upper jaw cartilages development (Wada et al., 2005; Eberhart et al., 2006). Although *gli1* expression did not appear affected in ventral brain primordium at 10 hpf in HMGCR loss-of function, we cannot discard that stomodeum formation and genes acting downstream of SHh, such as *pitx2* and *fgf8*, are affected (Eberhart et al., 2006; Schwend and Ahlgren, 2009). Further studies are needed to investigate this possibility.

Normal responses in activation of SHh signaling require cholesterol modifications (Porter et al., 1996; Steinhauer and Treisman, 2009), and several studies have demonstrated that accumulation of sterols and lack of cholesterol or/and its derivatives are responsible for defect in SHh signal activation in SLOS (Cooper et al., 2003; Blassberg et al., 2016). Our results suggested that, until the end of gastrulation, SHh signaling is apparently normal despite the HMGCR loss-of function, probably due to availability of cholesterol in yolk sac during early stage in zebrafish (Anderson et al., 2011; Fraher et al., 2016). Thus, the variability of craniofacial phenotypes that include cleft palate likely could be due to differences in the availability of maternal cholesterol in the yolk during early and late embryogenesis in zebrafish (Anderson et al., 2011; Fraher et al., 2016).

Similarly, in human and animal models of cholesterol synthesis disorders, differential maternal–embryonic transport and levels of cholesterol could partially explain the variability in the phenotype severity (reviewed in Woollett, 2008; Baardman et al., 2013). However, more studies are needed to test this hypothesis, such as measurement of the cholesterol and sterols in *hmgrb*$^{s617/s17}$ line and in pharmacological treatment with statin. For instance, others studies have shown that sterol depletion with cyclodextrin affects craniofacial morphogenesis in chicken embryos (Cooper et al., 2003). In addition, c.964-1G>C *Dhcr7* mutations and pharmacological treatment with BM15.766 (a specific inhibitor of DHCR7) induced cholesterol reduction causing holoprosencephaly and abnormalities characteristic of SLOS, independently of decreased oxysterols/increased sterols (Blassberg et al., 2016). Thus, the importance of increased sterols vs diminished cholesterol is still controversial in SLOS, and more studies will be required to determine specific effects of MVA intermediate metabolites and cholesterol on development of craniofacial malformation.

The role of HMCGR in the generation of orofacial malformations appears to be more complex than a simple disruption of SHh signaling. For example, *hmgcrb* mutant embryos presented delayed migration of CNCCs, and this process normally takes place in the absence of Hh signaling (Jeong et al., 2004; Eberhart et al., 2006; Schwend and Ahlgren, 2009). However, a recent study showed that SHh modulates through *cdon* TNCC migration in zebrafish (Powell et al., 2015). However, shortening of craniofacial structures, defects in intercalation, and variation in ceratohyal angle might be caused by disruption in chondrocyte morphology and convergent–extension movement during outgrowth of cartilage elements. Indeed, genetic and pharmacological impairment of HMGCR activity affects myocardial precursors and primordial germ cell migration and impacts the endothelial cells survival in zebrafish and *Drosophila* through defects in small GTPases prenylation-dependent processes (Van Doren et al., 1998; Santos and Lehmann, 2004; Thorpe

TABLE 4. *Expression of Craniofacial Genetic Markers in Embryos Treated with Atorvastatin during Early Stages of Orofacial Morphogenesis*

Genotype / phenotype	WT	DMSO 0.5%	Atorvastatin 5 μM
sox10 (24hpf)			
Normal	45 (100%)	62 (100%)	-
Mild reduction of expression in CNCCs with normal otic placode mRNA levels	-	-	42 (100%)
dlx2a (24hpf)			
Normal	222 (100%)	172 (100%)	-
Moderate decrease of expression in pharyngeal arches	-	-	159 (100%)
shha (48hpf)			
Normal	116 (100%)	187 (100%)	152 (92%)
Moderate shortening in domain extension of expression in oral ectoderm and pharyngeal endoderm	-	-	14 (8%)
gli1 (52hpf)			
Normal	42 (100%)	43 (100%)	-
Normal levels of expression with shortening in extension domain of expression in oral structures	-	-	59 (100%)
col2a1			
Normal	65 (100%)	63 (100%)	-
Mild shortening of domain expression in ethmoid plate	-	-	27 (56%)
Mild shortening of domain expression in lateral ethmoid and slight decrease of expression in medial ethmoid	-	-	21 (44%)

et al., 2004; Yi et al., 2006; D'Amico et al., 2007; Eisa-Beygi et al., 2013).

One possibility is that the decreased isoprenoids levels in HMGCR inhibition induce failure in prenylation of Rho and Rac small GTPases (Santos and Lehmann, 2004; D'Amico et al, 2007). Thus, we cannot reject that the phenotype found in our study might be produced by defects in the prenylation of small GTPases whose activity is very important in NCCs migration, proliferation, and death during craniofacial morphogenesis (Thomas et al., 2010; Liu et al., 2013) and in proliferation, apoptosis, morphology, and differentiation of chondrocytes (Woods et al., 2007; Wang et al., 2007; Aizawa et al., 2012). Furthermore, RhoA and Rac1 are Wnt/planar cell polarity signaling components that may regulate cartilage extension and outgrowth (reviewed in Szabo-Rogers et al., 2010; Topczewski et al., 2011). Future studies are needed to elucidate whether the spectrum of craniofacial phenotypes produced by disruption of HMGCR function may be induced also by defects in the small GTPase pathway.

In conclusion, the present study showed that impairment of HMGCR function has a key role in the induction of craniofacial malformation in zebrafish, differentially influencing early and late stages of orofacial development. Loss of HMGCR function produced a spectrum of craniofacial phenotypes that included OFCs, probably depending on MVA intermediate metabolites and cholesterol availability in the yolk sac, which can compensate for a lack of HMGCR function during early phases of craniofacial morphogenesis.

TABLE 5. *Pattern Expression of Craniofacial Genetic Markers in Embryos Treated with Atorvastatin during the Chondrogenesis Phase of Orofacial Development*

Genotype / phenotype	WT	DMSO 0.5%	Atorvastatin 5 μM
shha (48hpf)			
Normal	224 (100%)	300 (97%)	207 (88%)
Mild shortening in domain extension in oral ectoderm and pharyngeal endoderm	-	10 (3%)	27 (12%)
gli1 (52hpf)			
Normal	-	108	-
Severe reduction in ventral brain and moderate decrease of expression in oral structures with moderate shortening of domain expression	-	-	17 (15%)
Severe reduction of expression in ventral brain and moderate decrease of expression in oral structures with severe shortening of domain expression	-	-	99 (85%)
col2a1 (48hpf)			
Normal	294 (100%)	285 (100%)	-
Loss of more anterior ethmoid			13 (13%)
Absence of expression in ethmoid plate and short trabeculae	-	-	90 (87%)

In addition, our findings indicated that defects in HMGCR function affected mainly late phase of craniofacial development, presumably affecting postmigratory CNCC condensation and/or survival and chondrogenesis, in addition to producing defects in the normal morphology of cartilaginous skeleton. Defects in these cellular processes seems to be the result of an impaired SHh signaling pathway function but more detailed functional studies will be necessary to assess this possibility. It is important to consider that HMCGR impairment could be affecting other pathways, such as small GTPases, that may be also contributing to the craniofacial phenotype observed. Finally, this study raises several new questions about mechanisms involved in the induction of craniofacial malformations produced by MVA pathway defects and proposes a novel approach using zebrafish as a model to investigate inborn errors of cholesterol synthesis.

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