Control of Wnt5b secretion by Wntless modulates chondrogenic cell proliferation through fine-tuning fgf3 expression

Bo-Tsung Wu1,2, Shih-Hsien Wen1,2, Sheng-Ping L. Hwang3, Chang-Jen Huang1,2 and Yung-Shu Kuan1,2,4,*

ABSTRACT
Wnts and Fgfs regulate various tissues development in vertebrates. However, how regional Wnt or Fgf activities are established and how they interact in any given developmental event is elusive. Here, we investigated the Wnt-mediated craniofacial cartilage development in zebrafish and found that fgf3 expression in the pharyngeal pouches is differentially reduced along the anteroposterior axis in wnt5b mutants and wntless (wls) morphants, but its expression is normal in wnt9a and wnt11 morphants. Introducing fgf3 mRNAs rescued the cartilage defects in Wnt5b- and Wls-deficient larvae. In wls morphants, endogenous Wls expression is not detectable but maternally deposited Wls is present in eggs, which might account for the lack of axis defects in wls morphants. Secretion of endogenous Wnt5b but not Wnt11 was affected in the pharyngeal tissue of Wls morphants, indicating that Wls is not involved in every Wnt secretion event. Furthermore, cell proliferation but not apoptosis in the developing jaw was affected in Wnt5b- and Wls-deficient embryos. Therefore, Wnt5b requires Wls for its secretion and regulates the proliferation of chondrogenic cells through fine-tuning the expression of fgf3 during jaw cartilage development.

KEY WORDS: Wnt5b, Wntless, Fgf3, Wnt secretion, Chondrogenesis

INTRODUCTION
The secreted Wnt family proteins (Wnts) are key regulators of cell proliferation, differentiation, migration and apoptosis during the embryonic development of multicellular organisms. Many studies have provided rich insights into how cells receive and respond to Wnt signals (Baarsma et al., 2013; Bartscherer and Boutros, 2008; Logan and Nusse, 2004; Pronobis and Peifer, 2012). However, a comprehensive picture regarding how different molecules and mechanisms function with each Wnt pathway in any specific developmental process is far from being established. For instance, Wnts and Fgfs are known to regulate the development of skeletal tissues (Åberg et al., 2004; Baldridge et al., 2010; Bei and Maas, 1998; Brault et al., 2001; Church et al., 2002; Cuevas et al., 1988; Helms et al., 2005; Jentzsch et al., 1980; Liu et al., 2008; Reinhold et al., 2006; Rudnicki and Brown, 1997). Nevertheless, our knowledge on how Wnt and Fgf activities are present in a spatially and temporally regulated manner and how the chondrogenic and osteogenic cells integrate these two signaling activities to achieve the proper proliferation, differentiation or migration responses is still relatively limited.

In vertebrates, the β-catenin-mediated canonical and the non-canonical Wnt signaling pathways have both been shown to be involved in the processes of craniofacial skeleton formation. In mice, previous observations have indicated that Wnts can either stimulate chondrogenesis by promoting survival and differentiation of migrating neural crest cells (NCCs), or inhibit chondrogenesis by repressing BMP2-induced chondrocyte gene expression, depending on the developmental stage and the local tissue context (Brault et al., 2001; Liu et al., 2008; Reinhold et al., 2006; Yang et al., 2003). In zebrafish, wnt4a and wnt11r have been shown to regulate the formation of pharyngeal pouches, whereas wnt5b, wnt9a and wnt11 have been shown to regulate the development of pharyngeal arches and chondrocytes (Choe et al., 2013; Curtin et al., 2011; Heisenberg et al., 1996, 2000; Kimmel et al., 2001; Rauch et al., 1997). These findings indicate that Wnts mediate distinct effects on the generation and differentiation of chondrocytes. Therefore, to build a clear picture of how these Wnt molecules function to diversify the craniofacial skeletal plan requires the understanding of how different and active Wnt ligands are generated and exert their functions during the craniofacial cartilage development.

The transmembrane protein Wntless (Wls, also known as Zvi, Sprinter or GPR177) is one of the few proteins that have been identified to mediate the intracellular transport of Wnt molecules in both vertebrate and invertebrate cells. Since the discovery of Wls in 2001, studies have shown that Wls, as a transporter of Wnt ligands, binds Wnts in the Golgi and sends Wnts to the membrane surface for their release in Wnt-producing cells (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006), and that the retromer-complex-mediated membrane receptor recycling pathway is required for retrieving Wls from the plasma membrane back to Golgi (Belenkaya et al., 2008; Das et al., 2012; Eaton, 2008; Fanch-Marro et al., 2008; Pan et al., 2008; Port et al., 2008; Willert and Nusse, 2012; Yang et al., 2008). In Drosophila, Wls deficiency causes segmentation defects in larvae and wing-margin defects in adults. The homozygous mutants die before pupation (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). In mice, it has been shown that WLS is involved in diverse developmental processes, such as in body axis formation, in mammary, skeletal and pulmonary vascular development, and in adult hair follicle growth and regeneration (Cornett et al., 2013; Fu et al., 2009; Huang et al., 2012; Jiang et al., 2013; Maruyama et al., 2013a,b; Myung et al., 2013; Zhong et al., 2012; Zhu et al., 2013). However, although these WLS-deficient phenotypes have been associated with WNT-deficient phenotypes, whether WLS is involved in the secretion of all WNTs that mediate these developmental events nevertheless has not been clearly demonstrated to date. Because Wls-independent Wnt signaling has been found in Drosophila cells, and Wls-deficient zebrafish larvae show no obvious axial patterning defects or collective loss-of-Wnt phenotypes, it is possible that Wls might possess selectivity over...
different Wnt cargos (Bartscherer et al., 2006; Ching et al., 2008; Jin et al., 2010; Kelly et al., 1995; Matsui et al., 2005; Ramel et al., 2005; Westfall et al., 2003). However, whether those reported Wls-deficient fish embryos were in fact presenting a conditional loss-of-Wls phenotypes due to the presence of maternally inherited or derived Wls nevertheless should be carefully addressed.

Secreting Fgf family proteins (Fgf$s$), in addition to Wnts, have also been shown to play indispensable roles during the development of vertebrate craniofacial skeleton. In mice, FGF8 has been shown to induce Fgф3 expression in dental mesenchyme to regulate early tooth development (Åberg et al., 2004; Bei and Maas, 1998; Meyers et al., 1998). In zebrafish, Fgф3 expression in the pharyngeal pouch endoderm regulates the formation of branchial arch cartilages, and deleting Fgф3 along with Fgf8 enhances the Fgф3-deficiency-induced cartilage defects whereas deleting Fgf8 expression alone causes a lesser effect on the jaw cartilage formation (David et al., 2002; Hanaoka et al., 2004; Herzog et al., 2004; Nissen et al., 2003; Walshe and Mason, 2003). These essential and conserved roles of Fgf signaling pathways in vertebrate craniofacial skeleton development are reflected directly by the clinical discoveries that several human cranial facial skeletal abnormalities, such as Beare–Stevenson syndrome, Jackson–Weiss syndrome, Pfeiffer syndrome and otocephalodental syndrome, are caused by defective FGF signaling (Gregory-Evans et al., 2007; Ornitz and Marie, 2002; Passos-Bueno et al., 1999; Walshe and Mason, 2003). It is known that interplay between the Wnt and Fgf signaling pathways during various tissue formation processes occurs often during vertebrate development. For example, a negative feedback control between Wnt and Fgf signaling was found to be involved in zebrafish lateral line development, but a reciprocal positive control between Wnt and Fgf signaling was found to operate during zebrafish embryonic tail elongation (Aman and Piotrowski, 2008; Stubbberg et al., 2012). However, whether similar molecular programs between the components of the Fgf and Wnt pathways operate during craniofacial skeleton development are still unknown.

In this study, the molecular programs underlying the Wnt5b-mediated craniofacial cartilage development in zebrafish embryos were examined. Our analyses indicated that fgф3 expression in the developing pharyngeal pouches is differentially reduced along the anteroposterior axis in wnt5b mutants but is not reduced in wnt9a or wnt11 morphants. We also found that a similar fgф3 expression reduction and abnormal cartilage development were present in wls morphants. Introducing full-length fgф3 mRNAs could rescue the jaw cartilage defects caused by Wnt5b or Wls deficiency. Anti-Wls antibody staining showed that maternally deposited Wls was present at the one-cell and eight-cell stage, which might account for the lack of Wnt-associated axis defects in the Wls-deficient embryos. Immunohistochemical staining further indicated that endogenous Wnt5b secretion but not Wnt11 secretion required Wls. In addition, a reduction in cell proliferation was detected in the developing jaw of both Wnt5b- and Wls-deficient embryos. Therefore, our data reveal that Wnt5b requires Wls for secretion and that it regulates the proliferation of chondrogenic cells through fine-tuning fgф3 expression during embryonic jaw cartilage development. Our results also suggest that both Wls-dependent and Wls-independent Wnt signaling pathways are involved during the development of zebrafish jaw cartilage.

RESULTS

Ectopic expression of full-length fgф3 mRNAs rescued the jaw cartilage defects in wnt5b mutants

To understand whether Fgф3 is involved in the Wnt-mediated jaw cartilage development, we evaluated the fgф3 and fgф8 expression in wnt5b mutants and wnt9a or wnt11 morphants (Curtin et al., 2011; Lele et al., 2001; Rauch et al., 1997). The results showed that fgф3 expression in the developing pharyngeal pouches along the anteroposterior axis is reduced in wnt5b mutants but its expression is not reduced in wnt9a or wnt11 morphants at 48 h post-fertilization (hpf) (Fig. 1A–I). The expression of fgф8 is not different between wnt5b mutants and their wild-type (WT) siblings (data not shown). Therefore, these results suggest that Wnt5b controls jaw cartilage development by modulating fgф3 but not fgф8 expression. To test our premise, we introduced full-length in vitro-transcribed fgф3 and fgф8 mRNAs into wnt5b mutants. The results showed that introducing the full-length fgф3 mRNAs at 200 pg could rescue the jaw cartilage defects in the wnt5b mutant larvae whereas the tail elongation defects still remained in the injected wnt5b mutants (Fig. 1J–P). Morphological analyses of Alcian-Blue-stained samples indicated that the Meckel’s cartilages in wnt5b mutants did not extend as far anteriorly as the Meckel’s cartilages did in WT larvae, and the ceratohyal cartilages were shifted further posteriorly and became smaller in wnt5b mutants. In comparison, the position of the Meckel’s and ceratohyal cartilages, and the size of the ceratohyal cartilages, in the fgф3 mRNA-injected wnt5b mutants appeared to be normal (Fig. 1J–O). Given that the position shift of Meckel’s cartilages in wnt5b mutants resulted mainly from the malformation of the posterior cartilages (Fig. 1K,L), the rescue of wnt5b jaw cartilage phenotype by ectopically expressed fgф3 transcripts is therefore consistent with the previous report showing that fgф3 is mainly involved in the formation of posterior branchial arch cartilages (David et al., 2002).

To quantitatively evaluate the abnormality of these cartilaginous tissues, we analyzed the positional relationships between the Meckel’s cartilage, the two ceratohyal cartilages and the joints of the palatoquadrate and hyosymplectic cartilages by comparing the lengths of three reference lines (Line A, B and C in Fig. 1Q) that were defined as previously described (Goudevenou et al., 2011; see Materials and Methods). The results showed that the B to A (B:A) and C to A (C:A) ratios of the wnt5b mutants were statistically different from the ratios of the WT larvae. However, the B:A and C:A ratios of the fgф3 mRNA-injected wnt5b mutants were not significantly different from the ratios of the WT larvae (Fig. 1Q). Therefore, these data indicate that Wnt5b functions upstream to modulate the accumulation of the fgф3 transcripts along the anteroposterior axis during the development of jaw cartilages.

Wntless-deficiency caused jaw cartilage defects that resemble the defects in wnt5b mutants

Genes involved in the same molecular pathway during a tissue formation event are often found to cause similar tissue formation phenotypes. In this study, we found that, at 96 hpf, zebrafish wls morphants exhibited a prominent jaw defect resembling the jaw defect in wnt5b mutants, and their Meckel’s and ceratohyal cartilages were dislocated and smaller, as was observed in wnt5b mutants (Fig. 1L; Fig. 2A–J). In addition, we found that wls morphants also exhibited obvious ear and brain defects at 96 hpf but showed no obvious axis defect, and they survived to the extent that they were able to differentiate most of the larval organs at 48 hpf, even after a higher dose of morpholino (MO) injection (Jin et al., 2010). This was not expected because not only has the function of Wls been associated with the secretion of most, if not all, Wnts (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006), but Wnt signaling has also been shown to control the axis development in various vertebrates, including zebrafish, and axis
developmental defects have been observed in the Wls-knockout (KO) mice (Fu et al., 2009; Jin et al., 2010; Kelly et al., 1995; Matsui et al., 2005; Ramel et al., 2005; Westfall et al., 2003). Because the observed jaw defects in wls morphants did not represent the combined phenotypes from loss of Wnt4a, Wnt5b, Wnt9a, Wnt11 and Wnt11r (Choe et al., 2013; Curtin et al., 2011; Heisenberg et al., 1996, 2000; Rauch et al., 1997), we therefore hypothesized that the presence of maternally inherited Wls might help Wls morphants to bypass the requirement of Wls during early development, or that Wls might not be essentially required for the early stages of zebrafish embryonic development. At later stages, Wls might only control the secretion of a subset of Wnts during the development of jaw cartilages.

In order to visualize endogenously expressed Wls, we generated two polyclonal antibodies against recombinant proteins containing the C-terminal domain of zebrafish Wls. The whole-mount immunostaining patterns recognized by our anti-Wls antibodies recapitulated the expression patterns of the wls transcripts in embryos at 24, 48 and 72 hpf (Fig. 2K,M,P), except some non-specific signals were observed in the muscles where wls mRNA
expression was not detected (Jin et al., 2010 and our unpublished data). After MO treatment, the staining signals in the Wls-expressing domains disappeared in \( wls \) morphants, whereas the non-specific staining signals on muscles at 48 hpf were not affected; the staining signal of the endogenous Wls also disappeared too in our western blot analysis after MO treatment (Fig. 2O). These data indicate that our anti-Wls antibodies could recognize endogenous Wls, and that the \( wls \) MOs we applied could efficiently deplete Wls expressions by translational inhibition at 24 and 48 hpf (Fig. 2L,N,O). In addition, the \( wls \) MO we applied did not cause non-specific cell death as some other MOs do. Anti-Wls antibody staining of embryos at 0–4 hpf was conducted for evaluating Wls expression at earlier stages. The results showed that Wls proteins could be detected in eggs at one-cell and cleavage stages (Fig. 2P–S), indicating that the maternally deposited Wls are present in zebrafish eggs.

**Ectopic expression of full-length fgf3 mRNAs rescued the jaw cartilage defects in wls morphants**

To understand whether the jaw cartilage abnormality in the \( wls \) morphants was caused by the reduction in \( fgf3 \) expression, we examined the expressions of \( fgf3 \) and \( fgf8 \) at 48 hpf. The results showed that, similar to the \( wnt5b \) mutants, a reduction of \( fgf3 \) expression in the pharyngeal pouches was detected whereas the expression of \( fgf8 \) was not affected in the \( wls \) morphants at 48 hpf (Fig. 3A–G). We subsequently introduced full-length \( fgf3 \) mRNAs into the \( wls \) morphants and the results indicated that ectopically expressed \( fgf3 \) mRNAs could also rescue the jaw cartilage defects.

As can be seen, the positional relationships between the Meckel’s cartilage, the two ceratohyal cartilages and the joints of the palatoquadrate and hyosymplectic cartilages (B:A and C:A ratios in Fig. 1Q) in the \( fgf3 \)-mRNA-rescued larvae became no statistically
different from the control larvae (Fig. 3H–L). Therefore, similar to Wnt5b, Wls also controls jaw cartilage development through modulating the expression of fgf3 along the anteroposterior axis in pharyngeal pouches, suggesting that Wls is required for the secretion of active Wnt5b from its producing cells in the developing jaw.

Wntless is required for Wnt5b secretion

The results presented above have shown that, although Wnt5b, Wnt9a and Wnt11 exist simultaneously in the developing jaw, these Wnts possess different activities on regulating the Fgf3-mediated jaw cartilage development. The fact that Wls morphants do not exhibit collective loss-of-Wnt phenotypes but have a similar jaw defect to that in wnt5 mutants led us to hypothesize that Wls might not modulate the secretion of all the Wnts involved in the jaw cartilage development. Utilizing the antibodies available commercially, we examined the influence of Wls on the secretion of Wnt5b and Wnt11. The results demonstrated that, in the developing jaw of the control embryos, both Wnt5b and Wnt11 puncta were prominent near or on the cell membranes, indicating that these two Wnt proteins were in the process of secretion (Fig. 4A–K) (Chen et al., 2012). However, in the developing jaw of the Wls morphants, the Wnt5b puncta on the cell membranes had disappeared, whereas the Wnt11 puncta were still present on the cell membranes. This observation indicates that Wls controls Wnt5b but not Wnt11 secretion during the embryonic jaw development (Fig. 4L–Q).

Wntless controls chondrogenic cell proliferation during embryonic jaw cartilage development

When we were examining the jaw cartilage defects in the Wls-deficient larvae, we noticed that all cartilage elements were still present, yet these cartilages exhibited different degrees of size reduction although the individual cells in each cartilage looked fairly normal (Fig. 2A–F). We thus examined the expression of several marker genes to understand whether Wls-deficiency affected other Wnt-mediated development processes. The results showed that the expression of the neural crest cell (NCC) markers dlx2 and sox10, as well as the migrating lateral line primordium marker fgf3 and the canonical Wnt-responding gene dkk1b were normal in wls morphants or wnt5b mutants at 24 and 48 hpf (Fig. 5A–J). In addition, the chondrocyte and cartilage marker sox9a was expressed in the wls morphants at 96 hpf despite the fact that sox9a-positive cells were less-well distributed (Fig. 5K–P). These observations suggest that the cartilage abnormality likely results from abnormal cell death or proliferation rather than the defective specification or migration of the cranial NCCs or chondrocytes (Curtin et al., 2011; Yan et al., 2002). We therefore compared the cell apoptosis and proliferation status in wnt5b mutants and wls morphants utilizing a TUNEL assay, the anti-phosphohistone 3 (pH3) antibody staining and BrdU labeling (see Materials and Methods) (Berghmans et al., 2005; Shepard et al., 2004). The TUNEL assay showed that the apoptotic cell numbers in wls morphants and wnt5b mutants were not different statistically to those of MO control or WT siblings at both 24 and 48 hpf (7.4±2.2

![Fig. 3. Ectopic expression of full-length fgf2 mRNAs rescues the jaw cartilage defects in wls morphants.](image-url)
versus 7.3±1.9, P=0.916 and 4.6±1.3 versus 5.8±1.7, P=0.111 for wls; 7.8±1.9 versus 7.6±2.9, P=0.858 and 5.6±1.5 versus 5.8±1.9, P=0.806 for wnt5b, mean±s.e.m.) (Fig. 6A,B,G,H,M,N,S,T,Y,a,b; Fig. 7A,B,G,H,M,N,S,T,Y,a,b). By contrast, the cell proliferation assays showed that at 24 hpf, the pH3-positive cell numbers in wls morphants and wnt5b mutants were not different statistically to those in MO control or WT siblings (9.9±2.3 versus 10.1±1.9, P=0.89 for wls; 10.4±2.4 versus 10.2±2.3, P=0.857 for wnt5b), but they were statistically different at 48 hpf (51.7±5.3 versus 25.3±3.1, P=5.8156×10⁻¹¹ for wls; 53.2±5.4 versus 31.1±6.3, P=1.049×10⁻¹⁷ for wnt5b) (Fig. 6C,D,I,J,O,P,U,V,Y,c,d and Fig. 7C,D,I,J,O,P,U,V,Y,c,d). The reduced cell proliferation could be rescued by re-introducing fgf3 mRNAs into wnt5b mutants and wls morphants (supplementary material Fig. S1A–L). Similar to the anti-pH3 assay, the BrdU-positive cell numbers in wls morphants and wnt5b mutants were not different statistically to those in MO control or WT siblings at 24 hpf (55.4±6.6 versus 56.4±7.2, P=0.768 for wls; 53.9±8.2 versus 55.1±8.4, P=0.757 for wnt5b), but they were statistically different at 48 hpf (119.6±9.8 versus 81.2±11.3, P=1.9478×10⁻¹¹ for wls; 122±10.2 versus 85.9±11.4, P=6.3923×10⁻¹⁷ for wnt5b) (Fig. 6E,F,K,L,Q,R,W,X,Y,e,f and Fig. 7E,F,K,L,Q,R,W,X,Y,e,f). These data therefore indicate that Wls modulates chondrogenic cell proliferation to control the development of jaw cartilages. Because the change in proliferation frequency was only observed at 48 hpf and no cell death elevation was detected in the developing jaw in wls morphants, this thus indicates that the defective cartilage development in the wls morphants is not caused by p53-mediated cell death that has been associated with MO injection in some cases.

DISCUSSION
In this report, we have unraveled the regulatory relationship between Wls, Wnt5b and Fgf3, which has not been demonstrated previously during the development of craniofacial cartilages in vertebrates, despite the fact that many studies have provided rich insights into how cells receive and respond to individual Wnt or Fgf signals, and that defects in craniofacial skeleton development have previously been observed in vertebrates carrying mutations in Wnt or Fgf signaling components, including in humans (Baldridge et al., 2010; David et al., 2002; Gregory-Evans et al., 2007; Helms et al., 2005; Kimmel et al., 2001; Liu et al., 2008; Meyers et al., 1998; Ornitz and Marie, 2002; Passos-Bueno et al., 1999; Walshe and Mason, 2003; Yang et al., 2003).

Previously reports have shown that interactions between Wnt and Fgf signaling can be mediated by different mechanisms in different developmental processes given that negative-feedback control and
reduction of chondrogenic cells in the developing jaw, but specifically causes Wnt5b or Wls activities does not cause a general loss of the developing jaw to modulate molecular programs exist in the anterior and posterior ends of the embryo. This regional fine-tuning of Wnt5b gradient surrounding the pouch endoderm. This also explains why complete loss of Fgf3 causes jaw defects in wls morphants (Fig. 5E–H). These results thus indicate that loss of Fgf3 in the developing jaw was only partially downregulated by Wls and Wnt5b because fgf3 expression is partially reduced in wnt5b mutants and wls morphants, and re-introducing fgf3 mRNA rescues the jaw cartilage defects in both wnt5b mutants and wls morphants (Fig. 1). These results thus indicate that loss of Fgf3 or Wls activities does not cause a general loss of the chondrogenic cells in the developing jaw, but specifically causes reduction of fgf3 expression in the pharyngeal pouch endoderm that eventually leads to the abnormal jaw cartilage development. The rescue of jaw defects in wls morphants by fgf3 mRNA also indicates that Fgf3 secretion is not controlled by Wls in the developing jaw, which is consistent with the notion that the role of Wls is solely to modulate Wnt secretion (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). In zebrafish migrating lateral line primordia, fgf3 and fgf10 expression are both promoted by canonical Wnt signaling (Aman and Piotrowski, 2008). In this study, we found that the expression of fgf3 in the developing jaw was only partially downregulated by Wnt5b because the reduction of fgf3 expression was prominent in the anterior pouches but less obvious in the posterior pouches (Fig. 1B). This regional fine-tuning of fgf3 expression by Wnt5b but not by Wnt9a or Wnt11 (Fig. 1C,D) suggests that different molecular programs exist in the anterior and posterior ends of the developing jaw to modulate fgf3 expression in the pharyngeal pouch endoderm. This also explains why complete loss of Fgf3 caused a more severe jaw cartilage abnormality than the loss of Wnt5b or Wls does. It will be interesting to know whether the difference in fgf3 expression levels in different regions reflects the Wnt5b gradient surrounding the fgf3-positive pouches. Our results also suggest that Wnt5b, Wnt9a and Wnt11 do not function together or antagonize each other in the developing jaw, because alteration of fgf3 expression was not observed in the pharyngeal pouches of wnt9a and wnt11 morphants. This role of Wnt5b in jaw development is different from its role in axis formation where Wnt5b functions to antagonize canonical Wnt signaling (Westfall et al., 2003). In addition, although control of Wnt activity during lateral line development involves negative feedback from dkk1b induction, Wnt5b activity in jaw development does not seem to be regulated by dkk1b because a change of dkk1b expression was not observed in the pharyngeal tissue of wnt5b mutants (Fig. 5I,J).

Although the role of Wls was initially associated with the secretion of most if not all Wnts (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006), later studies have shown that Wls-independent Wnt secretion in fact exists in Drosophila cells (Ching et al., 2008). The observation that the jaw cartilage defects in wls morphants did not resemble the collective phenotypes from loss of Wnt4a, Wnt5b, Wnt9a, Wnt11 and Wnt11r, but instead resembled the cartilage abnormality observed in wnt5b mutants implies that Wls might control the secretion of only a subset of Wnts in the developing jaw of zebrafish. The fact that Wnt5b but not Wnt11 secretion is perturbed in cells in the developing jaw of the wls morphants indeed supports our hypothesis that Wls is not involved in every Wnt secretion event in vivo (Fig. 4). Whether the existence of Wls-dependent Wnt secretion identified in one type of cells can be extrapolated to another type of cell, however, requires further investigation. The existence of Wls-independent Wnt signaling is also supported by our observation that Wls might not involve in the canonical Wnt secretion during lateral line development because fgf3 expression in the migrating lateral line primordia is not affected by Wls deficiency (Fig. 5E–H). Alternatively, because maternally provided Wls proteins are present in eggs, this inherited Wls activity might be sufficient to support the Wnt-mediated processes such as planar cell polarity induction, axis formation and NCC specification and migration in the wls morphants at earlier developmental stages, hence explaining

**Fig. 5. Migration and specification of neural crest cells are normal in wls morphants.** (A–D) Dorsal views of 24 hpf control (5mis-wlsMO) (A,B) or wls-MO-injected (wlsMO) (C,D) embryos labeled with an antisense probe for dlx2 (A,C) or sox10 (B,D). (E–H) Lateral views of 24 hpf 5mis-wlsMO (E) or wlsMO (F) embryos labeled with the antisense fgf3 probes. Enlarged trunk regions from E and F are shown in G and H. Arrows indicate the migrating lateral line promodia. (I,J) Lateral views of 48 hpf WT (in I) or wnt5bMor (in J) embryos labeled with antisense dkk1b probes. (K–P) Images showing the sox9a expression in WT (K,L), wls 5mis-MO-injected (wlsMO) (M,N) and wlsMO (O,P) larvae at 96 hpf. Lateral views are shown in K,M,O; ventral views are shown in L,N,P. Numbers in the bottom left corners indicate the number of the embryos showing the indicated phenotype and the total number examined (denominator).
the lack of Wnt-associated defects in the \textit{wls} morphants found in this and the previous studies (Jin et al., 2010). The presence of maternally deposited Wls in zebrafish eggs, although meaning conditional loss-of-Wls embryos occur naturally, however, makes it technically challenging to generate maternal and zygotic loss-of-Wls embryos to evaluate the function of Wls at earlier developmental stages.

Functions of Fgf and Wnt signaling in growing embryos have been associated with cell proliferation, differentiation, migration and apoptosis in various developmental processes (Brault et al., 2001; Chi et al., 2003; Choe et al., 2013; Jászai et al., 2003; Rudnicki and Brown, 1997). In this study, we found that Wls modulates proliferation rather than survival of the chondrogenic cells during the development of jaw cartilages because a decrease in proliferation frequency was observed in \textit{wls} morphants at 48 hpf (Figs 6 and 7). These results suggest that canonical Wnt, Bmp and Fgf signaling are intact in the Wls-deficient embryos at the stages when cranial NCCs (CNCCs) are forming and migrating out from the neural plate border, because components of these signaling pathways have been shown to play crucial roles in the specification and migration of CNCCs (Choe et al., 2013; Garnett et al., 2012). Another thing worth noticing is that in addition to a prominent jaw defect, obvious brain defects were also present in the 96 hpf \textit{wls} morphants (Fig. 2B; Jin et al., 2010). Because alterations of both cell proliferation and apoptosis have been observed in the brains of the \textit{wls} morphants at 48 hpf (Fig. 7G), it therefore is conceivable that Wls plays a more complex role during the development of embryonic brain. This reasoning matches adequately with the function of Wls inside cells and the nature of the development of neural tissues where more diversified developmental processes and cell types are involved.

In summary, we have demonstrated that Wnt5b requires Wls for secretion and that it regulates the proliferation of craniofacial chondrogenic cells through fine-tuning the expression of \textit{fgf3} in the pharyngeal pouches (summarized in Fig. 8). Future studies focusing on how the \textit{fgf3} expression is controlled by Wnt5b signaling and whether there a feedback control is provoked by the Wnt5b-activated \textit{fgf3} expression will provide further knowledge on how Wnt and Fgf signaling interplay with each other in a \(\beta\)-catenin-independent manner. Our results also suggest that during the development of zebrafish jaw cartilage, both Wls-dependant and Wls-independent Wnt signaling pathways are involved. This new operational mechanism might be a general strategy in vertebrate cells to distinguish the different Wnts that are involved in given developmental process involving multiple Wnt signaling pathways.

**MATERIALS AND METHODS**

**Zebrafish lines and maintenance**

\textit{Danio rerio} AB strain and \textit{wnt5b} \textit{ti265} \textsuperscript{+} (pipetail) fish (Rauch et al., 1997) were obtained from Taiwan Zebrafish Core Facility at Academia Sinica (TZCAS). The \textit{wnt5b} ti265 allele is homozygous lethal. It therefore requires
crossing the \textit{wnt5b} heterozygous (+/−) parents to obtain the homozygous \textit{wnt5b} mutants (−/−) that in general would occupy 25% of the total progeny. All fish and embryos were raised and bred at 28°C based on the standard procedures described on ZFIN (http://zfin.org). All embryos were collected from natural spawning and kept at 28°C until the required experiment-performing stages. This work has been conducted following the Academia Sinica IACUC-approved protocol number RFiIBCKY2009084 and number 11-05-182.

Whole-mount \textit{in situ} hybridization and signal quantification

Whole-mount RNA \textit{in situ} hybridization (WISH) and riboprobe synthesis were carried out as described previously (Kuan et al., 2007). The clones for making anti-sense riboprobes of \textit{fgf3} (ZGC:194262), \textit{fgf8} (ZGC:101652) and \textit{dkk1b} (ZGC:152787) were purchased from the ATCC (American Type Culture Collection, USA). The full-length \textit{fgf8} and \textit{dkk1b} fragments (EcoRI and NotI sites) were further subcloned into a pCRII-TOPO vector (Invitrogen) for obtaining the polymerase sites. The clones for making anti-sense \textit{riboprobes of wls} (ZGC:64091), \textit{dlx2a} and \textit{sox9a} were gifts from Marnie Halpern (Carnegie Institution for Science, Baltimore, MD). The clone for making anti-sense \textit{sox10} riboprobes was generated by amplifying the \textit{sox10} cDNAs using total RNA from 24 hpf embryos, and the primer pair 5'-AACGCCAATTTCAGACCATAG-3' and 5'-ACCAAGGGAGACAATACAGAA-3', followed by TA cloning into the pCRII-TOPO vector. All images from WISH were taken with Canon EOS 600D on Leica DM RE microscope and were cropped to proper sizes for publication by Photoshop (CS5 edition, Adobe Systems). Signal intensities in the images showing enlarged pharyngeal tissues were quantified by ImageJ software (http://imagej.nih.gov/ij/) using the following steps: (1) convert the images into grayscale; (2) use the polygon tool to draw the bonding box; (3) eliminate background by adjusting the threshold; and (4) quantify the intensity by clicking ‘measure function’. All the statistical analyses and graphs shown in this study were performed or made in Microsoft Excel. Significance was evaluated by a two-tailed Student’s \textit{t}-test. Data are presented as mean±s.e.m. and differences were considered significant at \textit{P}<0.05.

\textbf{mRNA and antisense morpholino injection}

The clones for making full-length \textit{fgf3}, \textit{fgf8} and \textit{wls} mRNAs were the same full-length clones as used for probe making. Capped sense mRNAs were generated utilizing the mMESSAGE mMachine Kit (Ambion, USA). Antisense \textit{wls} MO (5'-CTCAATAATGGCCCCAGCATTTC-3') (Jin et al., 2010); \textit{wls} control MO (5mis-wlsMO) (5'-CTCAATAATGGCCCAATTTTT-3'), \textit{wnt9a} MO (5'-CCAGGAGAAGGTTCCATCCAGCAT-3'), and \textit{wnt11} MO (5'-GAAAGTTCCTGTATTCGTACATGGTC-3') (Lele et al., 2001) were purchased from Gene Tools (Philomath, OR). Approximately 50–200 pg of mRNAs and 2–8 ng of MOs in 1 nl solution were injected into embryos at the one- to two-cell stage (refer to the Results for the specific concentration for each experiment).
Cartilage staining and analysis

For cartilage visualization, Alcian Blue (Sigma, USA) staining was performed as described previously with minor modifications (Schilling and Kimmel, 1997). In brief, larvae were collected at 120 hpf and fixed in 4% PFA in PBS overnight then stored in PBS at 4°C before use. Alcian Blue (0.1%) dissolved in 0.37% HCl and 70% ethanol was used to label cartilages, and the stained samples were digesting with 0.02% trypsin for 2 h, then stored in 80% glycerol-KOH for visualization. All bright-field images were collected with a Canon EOS 600D (Japan) installed on a Leica DM RE microscope (Germany). To analyze the positional relationships of these cartilaginous tissues quantitatively, we adopted the method described previously (Goudevenou et al., 2011) with minor modifications. In brief, we compared the lengths between a baseline drawn between the joints of the palatoquadrate and hyosymplectic cartilages (line A in Fig. 1Q), the line drawn from the anterior end of the Meckel’s cartilage to the middle of the line A (line B in Fig. 1Q), and the line drawn from the anterior joint of the two ceratohyal to the middle of the line A (Line C in Fig. 1Q). The length of each line was measured by using ‘measure’ command in ImageJ.

Antibody generation and immunohistochemical staining

The clone for expressing recombinant Wls was generated by amplifying the cDNA using total RNA from 48–72 hpf embryos, and primer pair 5′-TGAGCTTCTTTG-3′ and 5′-GTGAGCTCTATTCT-TGAGCTTCTTTG-3′, followed by cloning into the pET28a vector (Novagen, USA) through the BamHI and SacI sites. The recombinant proteins were purified according to the method described previously (Lomasney et al., 1996). The anti-Wls containing sera were generated in rabbits or guinea pigs by commercial companies (GeneTex and Novagen, USA) through the BamHI and SacI sites. The recombinant proteins were purified according to the method described previously (Schilling and Kimmel, 1997). In brief, larvae were collected at 120 hpf and fixed in 4% PFA in PBS overnight then stored in PBS at 4°C before use. Alcian Blue (0.1%) dissolved in 0.37% HCl and 70% ethanol was used to label cartilages, and the stained samples were digesting with 0.02% trypsin for 2 h, then stored in 80% glycerol-KOH for visualization. All bright-field images were collected with a Canon EOS 600D (Japan) installed on a Leica DM RE microscope (Germany). To analyze the positional relationships of these cartilaginous tissues quantitatively, we adopted the method described previously (Goudevenou et al., 2011) with minor modifications. In brief, we compared the lengths between a baseline drawn between the joints of the palatoquadrate and hyosymplectic cartilages (line A in Fig. 1Q), the line drawn from the anterior end of the Meckel’s cartilage to the middle of the line A (line B in Fig. 1Q), and the line drawn from the anterior joint of the two ceratohyal to the middle of the line A (Line C in Fig. 1Q). The length of each line was measured by using ‘measure’ command in ImageJ.

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Proliferating and apoptotic cell labeling and quantification

Proliferating cells were labeled with rabbit anti-ph3 (see above) and BrdU (Sigma), and apoptotic cells were detected by using a cell death detection kit (TUNEL, Roche, Switzerland) as previously described (Berghmans et al., 2005; Shepard et al., 2004) with minor modification to the BrdU incorporation steps. In brief, a 5-min incubation with 5 mM BrdU in E3 buffer, mouse anti-BrdU (BD, USA) and goat anti-mouse-IgG conjugated to Alexa Fluor 568 (Invitrogen, USA) were utilized in our study. All staining experiments were performed along with the guinea pig anti-Wls antibody for distinguishing the Wls-deficient embryos from the normal embryos. The fluorescent images were acquired on a Leica SP5 laser confocal microscope. To count the labeled proliferating or apoptotic cells within the developing jaw cartilage, we have defined a regional block based on the z-axis interval (16–32 μm away from the surface), and the three lines drawn by the following rules: a parallel line drawn between the top point of the left eye to the top point of the otic placode (line 1 in Fig. 6Y and Fig. 7Y), a perpendicular line drawn through the posterior most point of the left eye (line 2 in Fig. 6Y and Fig. 7Y), and a tangent line drawn from the tangent point of the left eye to the tangent point at the border between the pharyngeal tissue and yolk (line 3 in Fig. 6Y and Fig. 7Y). Only those anti-ph3-, anti-BrdU- or TUNEL-positive cells residing in this defined regional block were counted in our experimental analyses.

Acknowledgements

We thank Taiwan Zebrafish Core Facility funded by the Ministry of Science and Technology, Taiwan (MST) grant number 103-2321-B-001-050 for fish line maintenance and reagents. We also thank Drs Masahiko Hibi (Nagoya U., Japan), Shinn Takeda (NINS, Okazaki, Japan), Geen-Dong Chang (NTU, Taiwan), Bon-Chu Chung (IMB, Academia Sinica) and Yun-Jin Jiang (NHRI, Taiwan) for critical maintenance and reagents. We also thank Drs Masahiko Hibi (Nagoya U., Japan), Shinn Takeda (NINS, Okazaki, Japan), Geen-Dong Chang (NTU, Taiwan), Bon-Chu Chung (IMB, Academia Sinica) and Yun-Jin Jiang (NHRI, Taiwan) for critical discussion, Dr Halpenny (Carnegie Institution for Science) for reagents, and Mrs. Ming-Chang Tsai, Yi-Chieh Chiu, Bob Fu, Dr Der-Yen Lee and Dr Sheng-Wei Lin for technical support.

Competing interests

The authors declare no competing or financial interests.

Author contributions


Funding

This work has been supported by Ministry of Science and Technology, Taiwan (MST) [grant number 103-2321-B-001-050 for fish line maintenance and reagents]. We also thank Drs Masahiko Hibi (Nagoya U., Japan), Shinn Takeda (NINS, Okazaki, Japan), Geen-Dong Chang (NTU, Taiwan), Bon-Chu Chung (IMB, Academia Sinica) and Yun-Jin Jiang (NHRI, Taiwan) for critical discussion, Dr Halpenny (Carnegie Institution for Science) for reagents, and Mrs. Ming-Chang Tsai, Yi-Chieh Chiu, Bob Fu, Dr Der-Yen Lee and Dr Sheng-Wei Lin for technical support.


Fig. S1. Introducing full-length fgf3 mRNAs rescue the cell proliferation defects in wnt5b and wls-deficient embryos. (A-D) Confocal images showing lateral view of the heads of wls MO and fgf3 mRNA co-injected embryos stained by anti-Wls (green in A, C) plus anti-pH3 (red in A-B) or anti-BrdU (red in C-D). (E-H) Confocal images showing lateral view of the heads of and fgf3 mRNA-injected wnt5b mutant embryos stained by anti-Wls (green in E, G) plus anti-pH3 (red in E-F) or anti-BrdU (red in G-H). The dot-lines of each triangle are defined as described in Material and methods and Fig. 7Y. (I-L) Statistical charts represent the quantitative results of proliferating cells (pH3 or BrdU positive) at 48 hpf. Mark ”No.” denotes numbers. Mark “cMO” in denotes 5mis-wlsMO. Mark “+fgf3” denotes fgf3 mRNA-injected. Symbols “*” indicates statistically indifferent and “**” indicates statistically different (see Materials and methods).