Modulation of tooth regeneration through opposing responses to Wnt and BMP signals in teleosts

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Abstract

Most vertebrates are capable of regenerating entire tooth organs. Tooth regeneration has long been hypothesized to rely on extracellular signals that can influence multiple tooth sites at once. Little is known about which secreted signaling molecules can influence this process. As an entry point, we asked whether fish orthologs of genes known to regulate mammalian hair regeneration have effects on tooth regeneration or total tooth number. We tested whether tooth regeneration could be accelerated by exogenous Wnt signaling (via Wnt10a) or BMP inhibition (Grem2a), and if regeneration rates were slowed by exogenous BMP signaling (Bmp6) or Wnt inhibition (Dkk2). Using two fish species that demonstrate distinct modes of whole tooth regeneration, the threespine stickleback (Gasterosteus aculeatus) and zebrafish (Danio rerio), we found that transgenic overexpression of four different genes changed tooth replacement rates in the predicted direction: Wnt10a and Grem2a increased the tooth replacement rate, while Bmp6 and Dkk2 strongly inhibited replacement tooth formation. Regulation of total tooth number was separable from regulation of regeneration rates. In zebrafish, none of the factors affecting regeneration rate affected the number of distinct tooth families but did sometimes affect total tooth number. In sticklebacks, which do not exhibit clear tooth families, Bmp6 and Dkk2 reduced total tooth number, while Wnt10a and Grem2a did not. These data support a model where different epithelial organs like teeth and hair share genetic inputs driving the timing of whole organ regenerative cycles.
Introduction

Tooth regeneration is a trait demonstrated by most vertebrate groups\textsuperscript{1–3}. This regenerative process usually takes the form of whole tooth replacement, where entire new tooth organs differentiate while old teeth are removed by active shedding and/or dislodgement through use. Tooth replacement events often exhibit a high degree of within-species consistency with respect to their timing, sequence, and spacing, forming characteristic replacement patterns. This consistency has led to hypotheses that neighboring or alternating tooth positions could be subject to signals that act to influence or coordinate the timing of regeneration cycles for multiple teeth in tandem\textsuperscript{4–9}. The potential involvement of secreted signals in tooth regeneration is additionally suggested by studies on primary tooth differentiation, where the functional relevance of numerous signaling ligands has been documented\textsuperscript{10–22}. However, little is known about which secreted signals can regulate the process of whole tooth regeneration.

Whole organ regeneration is a trait that teeth share with many other epithelial appendages. This class of organs includes body coverings like hair, scales, and feathers, and some soft organs like salivary glands and sweat glands\textsuperscript{23–25}. Whether regeneration is cyclic and programmed or only brought on by injury or wear, nearly all types of epithelial appendages can be wholly replaced via regeneration\textsuperscript{26–33}. Despite the stark differences in their basic compositions as mature organs, different epithelial appendages demonstrate numerous developmental genetic similarities, in some cases suggesting deep homology or even direct homology between these organs\textsuperscript{24,34–41}. Given that most epithelial organs demonstrate the capacity to regenerate, we parsimoniously hypothesize that these regenerative processes are driven by shared genetic networks. We previously tested this idea by looking at fine-scale gene expression during zebrafish and stickleback tooth regeneration\textsuperscript{42}. This study found that ten hair follicle stem cell marker candidate genes demonstrated orthologous gene expression in both zebrafish and stickleback successional dental epithelia (SDE), including CD34, Gli1, Nfatc1, and
Bmp6. This battery of gene expression marks the epithelia presaging tooth and hair regeneration in their respective cellular contexts, suggesting a remarkable level of genetic overlap in these naïve tissues.

In the present work, we aimed to test whether genetic mechanisms known to drive hair regeneration could similarly influence tooth regeneration in sticklebacks and zebrafish. In mammalian hair, some secreted factors have been identified as likely or possible regulators of hair follicle stem cells. Namely, a Wnt-BMP cycling mechanism has been well supported, whereby the Wnt and BMP pathways have oppositional roles that drive the oscillation of the hair regenerative cycle between active growth (anagen, high Wnt + BMP inhibitors) and quiescence (telogen, high BMP + Wnt inhibitors)\textsuperscript{26,43–45}. Ligands implicated in promoting hair regeneration include Wnt ligands, Wnt10a, Wnt10b, Wnt7a, and Wnt7b, as well as specific BMP inhibitors Grem1, Grem2, Bambi, and Noggin\textsuperscript{11,43,44,46–49}. Conversely, secreted BMP signals like Bmp2, Bmp4, Bmp6, and Wnt inhibitors like Dkk1 and Dkk2 have been implicated in slowing or stopping the regenerative process\textsuperscript{26,43,44,50–52}. To test whether such secreted ligands could elicit congruent changes in the regeneration rates of teeth, we selected four of the above genes (Wnt10a, Dkk2, Bmp6, and Grem2a) to test for both endogenous expression in actively regenerating tooth fields, and their possible effects on both tooth replacement rates and total tooth number. Our selection of specific gene orthologs was partly motivated by known pleiotropic disease loci in humans: WNT10A and GREM2 are known to be associated with different forms of ectodermal dysplasia, where both tooth and hair regeneration are perturbed, but not always primary epithelial organ growth\textsuperscript{11,53}. Grem2 loss-of-function has been shown to disrupt constant incisor outgrowth in mice\textsuperscript{14}. Bmp6 has been strongly implicated in both mouse hair regeneration\textsuperscript{43,44} and the natural evolution of stickleback tooth regeneration rates and total tooth number\textsuperscript{54,55}. Dkk2 expression has been shown to oscillate during the regenerative cycle in the hair follicle\textsuperscript{52} and is sufficient to cause ectopic hair to grow in normally hairless regions of
mouse skin\textsuperscript{56}. All four of these selected genes demonstrate substantial expression levels in a previously published RNAseq dataset derived from late-stage stickleback tooth fields undergoing regeneration\textsuperscript{57}, suggesting these genes could regulate tooth regeneration. Furthermore, stimulation of downstream Wnt signaling via a constitutively active $\beta$-catenin ($\textit{Ctnnb}$) has been shown to induce replacement events in mouse molars, a process which normally does not occur in this species, suggesting that Wnt upregulation indeed plays a crucial role in promoting whole tooth organ regeneration\textsuperscript{58,59}.

**Results**

**Expression of secreted ligand genes of interest in stickleback**

We examined $\textit{Wnt10a}$, $\textit{Dkk2}$, $\textit{Bmp6}$, and $\textit{Grem2a}$ expression in wild-type subadult stickleback pharyngeal tooth fields (Fig. 1). We documented expression not just in tooth organs themselves, but also the regions between teeth, as these could also be involved with regulating tooth organ development or regeneration. Overall, we found that all four genes are expressed both in developing tooth organs and in epithelial and/or mesenchymal cell populations surrounding tooth organs.

$\textit{Wnt10a}$ transcripts were detected in early bud-stage tooth germs in both the epithelium and the earliest tooth mesenchyme, as previously published (Fig. 1A). Epithelial expression was also observed at the late-bell stage, favoring the inner dental epithelium (Fig. 1B-D). $\textit{Wnt10a}$ Expression in dental mesenchyme persists through eruption (Fig. 1C) but is not appreciably detected in fully ankylosed and erupted teeth (Fig. 1D). $\textit{Wnt10a}$ is also detected in isolated regions of epithelial and mesenchymal cells at other locations in the tooth field, most often in mesenchyme near or below developing tooth germs (Fig. 1A-C, brackets).
The Wnt signaling inhibitor Dkk2 was diffusely expressed in the epithelium overlying the entire tooth field (Fig. 1E and F). Increased epithelial expression detected in bell-stage tooth organs, especially in the inner dental epithelium (Fig. 1E). In tooth mesenchyme, we first detected expression in the early bell stage (Fig. 1E), which appears to persist through tooth differentiation, including in odontoblasts located towards the apex (tip) of the tooth in fully ankylosed and erupted teeth (Fig. 1F). Dkk2 was additionally observed in deep mesenchymal cell populations between teeth, against the bone of attachment that serves as the anchor for ankylosed teeth (Fig. 1E, bracket).

Bmp6 was expressed similarly to Wnt10a in bud-stage teeth, exhibiting focal expression in both the epithelium and mesenchyme (Fig. 1G). As previously published, Bmp6 was detected in the inner dental epithelium and mesenchyme of bell-stage tooth germs (Fig. 1H), and mesenchymal expression is maintained during and after eruption (Fig. 1I and J). Bmp6 expression was detected at a subset of successional dental epithelium (SDE) locations as well as isolated clusters of mesenchymal cells surrounding teeth in the tooth field (Fig. 1I).

The BMP inhibitor Grem2a was detected in mesenchyme adjacent to bud stage tooth germs but appeared to be excluded from the epithelium and dense mesenchymal condensation of the early tooth germ itself (Fig. 1K). During bell stages, tooth germs exhibited both inner and outer dental epithelial expression as well as mesenchymal expression (Fig. 1L and M), all of which was also present in eruption stage teeth (Fig. 1M). Fully ankylosed and erupted teeth maintained mesenchymal expression in odontoblasts (Fig. 1M). Grem2a transcripts were additionally detected in the SDE, in other dispersed epithelial cells (Fig. 1M), and in mesenchymal cells surrounding teeth and tooth organs in the tooth field (Fig. 1K-M).
Pulse-chase bone labeling and gene overexpression approach

Three of the four secreted ligand genes we focus on here are required for normal primary tooth development in different species (\textit{Wnt10a}\textsuperscript{10,11}, \textit{Bmp6}\textsuperscript{55}, and \textit{Grem2a}\textsuperscript{14,53}). Such early requirements present obstacles to using germline loss-of-function mutations to understand gene functions in late-stage tooth fields, because alterations to early primary tooth differentiation likely confound interpretations of later events like regeneration. We thus sought a genetic system that could test the effects of secreted Wnt and BMP pathway members of interest during late developmental stages without interfering with tooth field initiation and early primary tooth differentiation. Heat shock gene overexpression (OE) is as attractive option because it is temporally inducible during late developmental timepoints and well established in zebrafish\textsuperscript{60,61}. The strategy we used thus couples OE treatments of \textit{Wnt10a}, \textit{Dkk2}, \textit{Bmp6}, or \textit{Grem2a} with a two-color pulse-chase bone staining protocol (summarized in Fig. 2), allowing us to classify each tooth in each individual as either “new” or “retained” with respect to the OE treatment interval (see Methods). By calculating the new:retained tooth ratio for each fish, we invoke a simple proxy of each individual’s “tooth replacement rate,” which addresses whether changes to the pace of the regenerative cycle have occurred. For both species, we sum all new and retained teeth to assess “total tooth number,” a count that includes both erupted functional teeth and unerupted bony tooth germs. In sticklebacks this encompasses both oral and pharyngeal teeth usually numbering \(\sim200-300\), whereas zebrafish have just one pair of pharyngeal tooth fields with \(\sim25-30\) total teeth. Since zebrafish exhibit morphologically stationary tooth families with a stereotypical number and arrangement that is reached during early juvenile stages (\(\sim30\) days old)\textsuperscript{62}, we additionally ask whether OE treatments can modify the number of tooth families present in zebrafish.
Wnt pathway modulation by overexpression of Wnt10a and Dkk2

We first sought to test whether positive regulation of the Wnt pathway could influence tooth regeneration or total tooth number. Due to the known requirement of WNT10A in human EA development and regeneration, and expression in early tooth fields across many vertebrates, we asked whether Wnt10a is sufficient to promote tooth replacement rate or total tooth number in stickleback and zebrafish (Fig. 3A). Wnt10a OE in sticklebacks simultaneously increased the number of new teeth \((P=0.00072)\) and reduced the number of retained teeth \((P=0.01)\), consistent with a role in promoting tooth regeneration. Together, these two shifts consistently raised the average new:retained tooth ratio \((P=0.0003)\), as predicted. However, Wnt10a OE did not significantly change the total number of teeth \((P=0.81)\), demonstrating that changes to the regeneration rate don’t necessarily alter changes to total tooth number. These shifts are also generally reflected by most tooth field types alone (Fig. S1), suggesting generally consistent effects of this Wnt signaling ligand in both oral and pharyngeal tooth fields. Qualitatively, we noticed that the Wnt10a OE individuals oftentimes displayed uninterrupted clusters of five or more new teeth (Fig. 3C, dotted oval), whereas new tooth distribution in wild-type (WT) controls appeared more uniform.

We sought to determine whether the Wnt10a OE transgene could affect tooth replacement rates or total tooth number in carrier animals raised at normal temperatures. To test for these effects, we performed a negative control pulse-chase assay with the same staining interval (18 days) but with no intervening heat shocks, raising similar numbers of WT and transgene-carrying fish in the same tanks/conditions as the heat shock treatment but kept at normal temperatures (~17° C). Notably, these fish were full siblings to the treatment animals described above, helping to control for genetic variation. We found no significant deviation in the number of new teeth, number of retained teeth, the new:retained tooth ratio, and the total tooth number (Fig. S2).
We next tested \textit{wnt10a} OE in zebrafish (Fig. 3D). Using the same 18 day pulse-chase overexpression assay, but with zebrafish resting and heat shock temperatures (see Methods and Fig. 2B), we found an increase in the number of new teeth formed during the treatment interval (\(P=0.00039\)). However, unlike sticklebacks, the number of retained teeth was unchanged (\(P=0.56\)). Overall the new:retained tooth ratio was significantly increased (\(P=0.0042\)). While we did not observe any change in the number of tooth families in any OE individual (\(n=15/15\)), we did find an increase in the total tooth number under \textit{wnt10a} OE (\(P=0.011\)), due to a higher number of tooth families undergoing early replacement.

To address whether Wnt signaling inhibition could negatively influence tooth growth, we asked if Dkk2, a secreted Wnt signaling inhibitor, could decrease tooth replacement rates or total tooth number in sticklebacks (Fig. 4A). We found that Dkk2 OE strongly reduced the presence of new teeth (\(P=0.00024\)), while simultaneously increasing the presence of retained teeth (\(P=0.045\)). Together, these two effects led to a sharp decrease in the new:retained ratio (\(P=1.1e^{-5}\)), while also decreasing the number of total teeth (\(P=0.0036\)). In the control condition, new teeth comprised mostly mid- or late bell stage tooth germs (Fig. 4B, arrows), whereas the few unankylosed new teeth we observed under Dkk2 OE were always at or near the eruption stage (Fig. 4C, arrow). These results are also generally reflected by each tooth field type alone (Fig. S3), although the oral tooth fields show a generally weaker response. To assess whether Dkk2 OE could be stalling tooth germs at stages prior to bone deposition (at bud, cap, or early-bell stages), we analyzed tooth field histology using H&E-stained sections (Fig. 4D and E). We found no evidence of bud, cap, or early-bell stage tooth germs across any pharyngeal tooth field we observed from Dkk2 OE individuals (\(n=7/7\)), suggesting that Dkk2 does not cause existing tooth germs to arrest during early differentiation.
BMP pathway modulation by overexpression of Bmp6 and Grem2a

We next sought to test whether modulation of Bmp signaling could affect tooth regeneration rates or total tooth number. Specifically, our hypothesis of a hair-like cycling mechanism operating in teeth predicts that tooth replacement should be inhibited by increases in BMP signaling and promoted by BMP inhibition. To test this hypothesis, we conducted a Bmp6 OE treatment, given known roles for Bmp6 in EA development and regeneration, including in stickleback teeth and mouse hair. Bmp6 OE produced a striking tooth phenotype unique among the phenotypes observed in this study: late bell (bony) tooth germs that were negative for both Alizarin Red and calcein (Fig. 5). We interpret these unstained tooth germs as “stalled,” i.e. tooth germs that initiated bone growth just after the Alizarin pulse/treatment onset, but ceased differentiation and bone deposition during the treatment interval and were thus no longer producing bone by the end of the treatment and the calcein chase. We conservatively counted these unstained, stalled tooth germs as assumed “new” teeth for this treatment, which were unique to Bmp6 OE and never otherwise observed. Even so, Bmp6 OE resulted in sharply reduced new tooth formation ($P=2.7e-5$, Fig. 6A). Surprisingly, Bmp6 OE also resulted in a decrease in retained teeth ($P=2.8e-5$), suggesting that Bmp6 negatively affects new tooth formation while also promoting the shedding of existing teeth. Despite both new and retained teeth being reduced, Bmp6 OE led to a decrease in the new:retained tooth ratio ($P=4.4e-5$), indicating that exogenous Bmp6 did overall inhibit the tooth replacement rate by more strongly decreasing the number of new teeth present relative to retained teeth. Given that we found a drop in both new and retained teeth, Bmp6 OE necessarily caused a drop in total tooth number ($P=2.8e-5$), resulting in the treatment group having only ~50-60% of the number of teeth that their WT control siblings possessed at the end of the OE treatment. Most of these trends are reflected by each tooth field type alone (Fig S4). Qualitatively, we found large swaths of
stickleback tooth fields that were devoid of erupted teeth (Fig. 5, dotted oval), which was unique to this treatment condition.

We additionally performed a negative control assay on the Bmp6 OE line, again allowing us to ascertain whether carrying the transgene without heat shocks is sufficient to alter any aspects of tooth turnover, total tooth number, and in this case, the tooth “stalling” we uniquely observed under Bmp6 OE. As with the Wnt10a negative control experiment, we selected full siblings to the Bmp6 OE treatment animals, and pulse-chased them with no heat shocks in the same containers as our heat shock treatments. We again found no significant deviation in any variable between either pair of groups (Fig. S5). Notably, we found no calcein negative tooth germs in this negative control experiment. In this case, the variance in the non-WT groups did not appear to be increased relative to WT.

We next asked if bmp6 OE had any of these same effects on zebrafish tooth fields (Fig. 6D). While we did not observe any stalled tooth germs as we did in sticklebacks, we did find a significant decrease in the number of new teeth that formed during the treatment interval ($P=0.0042$), consistent with a negative role in tooth germ initiation and/or differentiation. Unlike sticklebacks, zebrafish exhibited an increase in retained tooth number ($P=0.78$). The new:retained ratio, resulting from both an increase in new and decrease in retained, also fell significantly ($P=0.013$). We did not observe any changes to the number of tooth families present ($n=9/9$). However, we did detect a significant decrease in the overall number of teeth ($P=0.0035$), likely brought on by the paucity of new bell stage tooth germs in the OE condition.

To test whether BMP pathway inhibition could promote tooth turnover, we performed an overexpression experiment using the BMP antagonist Grem2a in sticklebacks (Fig. 7A). Grem2a OE did not significantly increase new tooth number ($P=0.64$) but did decrease retained tooth number ($P=0.039$). This drop in the retained tooth number per fish was sufficient to increase the new:retained tooth ratio ($P=0.029$), achieving a higher relative proportion of new
teeth by eliminating retained teeth. However, unlike Bmp6, which also decreased the number of retained teeth, Grem2a OE did not significantly reduce total teeth ($P=0.15$). These results are also generally reflected by each tooth field type alone (Fig. S6).

We finally tested grem2a OE in zebrafish to ask whether the BMP signaling inhibition also promoted tooth replacement in this species (Fig. 7B-D). This treatment resulted in an increase in the new:retained tooth ratio ($P=0.00013$), which arose from a simultaneous increase of new teeth ($P=0.00031$) and a drop in retained teeth ($P=0.0047$). Zebrafish grem2a OE did not alter the number of tooth families observed ($n=11/11$), nor did it cause a significant change in the total number of teeth ($P=0.14$).

Discussion

Endogenous expression domains of stickleback Wnt10a, Dkk2, Bmp6, and Grem2a reflect similar germ layer partitioning as in hair follicle gene expression.

We first used in situ hybridization to analyze the endogenous expression patterns of Wnt10a, Dkk2, Bmp6, and Grem2a in subadult stickleback tooth fields. Our use of sticklebacks allows for a detailed analysis of tooth germ differentiation using only 3-5 individuals per gene, as each subadult stickleback individual (~25 mm standard length) presents ~30+ tooth germs in total. By comparing gene expression between stickleback tooth germs of different observed stages we infer a general tooth organ staging series for each gene, which we can use to interpret whether any expression domains are likely cyclic during the tooth regeneration cycle. As a hair regeneration model would predict, Wnt10a, Dkk2, Bmp6, and Grem2a exhibit expression in tooth epithelium and tooth mesenchyme that appears somehow cyclic during tooth organ regeneration. These four genes additionally show expression partitioning between epithelial and
mesenchymal germ layers in ways that overall resemble expression of the mammalian orthologs of these genes in hair follicles (detailed below).

*Wnt10a* was focally expressed in both epithelium and mesenchyme of developing tooth germs, but epithelial expression appeared to wane by late bell stages, and mesenchymal expression was essentially absent in erupted tooth mesenchyme (Fig. 1A-D). This expression roughly corresponds to the cycle previously observed in the mouse hair follicle, where *Wnt10a* expression was found in the early anagen (growth) phase, but not telogen (the quiescent resting phase which ends with hair shedding)\(^3\). The Wnt inhibitor *Dkk2* is thought to confer a feedback response in mouse hair follicles, wherein its expression in hair follicle mesenchyme (the dermal papilla) is brought on by Wnt effectors and helps trigger the end of the growth (anagen) phase\(^2\).

Stickleback mesenchymal expression domains of *Wnt10a* and *Dkk2* are generally consistent with such a relationship: *Wnt10a* shows marked expression at the beginning of tooth differentiation (bud stage) that wanes throughout differentiation (Fig. 1A-D, whereas *Dkk2* is excluded from bud stage mesenchymal cells, transcribed during bell stages, and is then highly expressed during and after eruption, especially in fully differentiated apical tooth mesenchyme (odontoblasts; Fig. 1E and F).

BMP pathway genes additionally show expression domains in stickleback tooth fields that are partitioned by germ layer and stage in a way that roughly corresponds to mouse hair follicles. *Bmp6* is highly expressed during growth (anagen) stages of the mouse hair follicle cycle\(^4\), similarly to the inner dental epithelial and mesenchymal expression that is present during early tooth germ growth\(^42,54\) (Fig. 1H). Despite this marked expression of *Bmp6* during organ differentiation, hair follicles have been shown to be delayed from initiating organ development by inhibiting the telogen to anagen transition\(^4\). Broad similarities exist with teeth: in both stickleback and zebrafish, *Bmp6* demonstrates marked upregulation in specific dental tissues during tooth organ differentiation\(^42\), while also preventing the formation of new tooth
organs (Fig. 6A and D). *Grem2* expression has not been analyzed in mouse hair to our knowledge, but *GREM2* expression in human hair follicles is sharply restricted to the mesenchymal dermal sheath cup, which has led to hypotheses that it regulates the hair follicle stem cell niche\(^{46,64}\). Similar to human *GREM2*, stickleback *Grem2a* expression was more widespread in mesenchymal cells in general, however was also more widely expressed than in human hair fields (Fig. 1K-M), including expression in mid-bell stage tooth epithelium and mesenchyme (Fig. 2L), as well as the SDE (Fig. 2M).

Overall, we found that the dental mesenchyme and inner dental epithelium of bell stage tooth germs expresses all four of these secreted ligands. Previous results in sticklebacks and other vertebrate species have described additional expression patterns of an array of Wnt and BMP ligands, inhibitors, and receptors in and around teeth and tooth fields\(^{39,42,54,65–79}\). The apparently high representation of different secreted ligands suggests that tooth fields are subject to a rich amalgam of Wnt and BMP signaling molecules overall. It is thus likely that the final output of Wnt or BMP signaling in a given cell might ultimately be regulated by dozens of different secreted ligands and inhibitors all dynamically competing in the extracellular space within the tooth field\(^{80}\). The endogenous expression patterns of these secreted factors in tooth fields additionally leaves open the possibility that they participate in some form of reaction-diffusion system between tooth organs and/or more broadly as part of some type of regulatory centers or waves that can coordinate tooth regeneration. Both reaction-diffusion and regulatory wave mechanisms have been hypothesized to explain different coordinated aspects of hair field growth or regeneration\(^{26,81–84}\). Intriguingly, some studies have even found specific roles for Wnt and BMP signals in these mechanisms, including *Dkk*\(^{26,81}\). Nevertheless, the four genes we focused on here are all expressed both in tooth germs themselves, as well as in some epithelial and mesenchymal domains that are outside of differentiating or functional tooth organs (Fig. 1, brackets). Any of these expression domains, including those in the “intertooth spaces,” may
work to influence tooth regeneration or total tooth number. Loss of function studies for *Wnt10a*, *Dkk2*, *Bmp6*, and *Grem2a*, including future tissue-specific ablation (e.g. in tooth germs vs. intertooth space) could help determine whether these genes are required for tooth field morphogenesis or regeneration.

**Opposing roles of the Wnt and BMP pathways in epithelial appendage regeneration**

Wnt and BMP signals have well-documented roles in attenuating hair regeneration in mice. The progression of mouse hair regeneration has been shown to be promoted by Wnt ligands and BMP inhibitors, while inhibited by BMP ligands and Wnt inhibitors\(^{26,43-45,50,85}\). This Wnt-BMP oppositional mechanism regulates the cyclic regeneration of hair organs. We reasoned that different epithelial appendages, including teeth, could use a similar basic input scheme to dictate the progression of regeneration, overall being positively influenced by Wnt, and negatively influenced by BMP. To test the hypothesis that teeth and hair share instructive functions for specific secreted ligands during regeneration, we used a candidate gene approach to ask whether specific Wnt and BMP pathway members could regulate tooth regeneration rates in a manner congruent with their known or suspected roles in the Wnt-BMP mechanism described in hair follicles. We thus developed a genetic overexpression (OE) system that could test whether *Wnt10a*, *Dkk2*, *Bmp6*, and *Grem2a* were sufficient to regulate tooth regeneration and/or tooth number overall. To access this in OE treatments on adult and sub-adult organisms actively undergoing tooth regeneration, we measured the number of “new” and “retained” teeth in each fish, calculated a replacement rate of teeth via the new:retained tooth ratio, and also calculated a total tooth number. Our hypothesis specifically predicted that Wnt pathway stimulation or BMP pathway inhibition would increase the replacement rate of teeth, while BMP pathway stimulation or Wnt pathway inhibition would inhibit regeneration. Overall, our results were consistent with our hypothesis: we found that *Wnt10a* or *Grem2a* OE increased tooth
replacement rates in both sticklebacks and zebrafish, while Bmp6 or Dkk2 OE both decreased the initiation of new teeth, the former in both sticklebacks and zebrafish, the latter in sticklebacks only (we did not test zebrafish dkk2 OE). Our overall interpretation of these results is that tooth regeneration is indeed modulated in a congruent fashion as hair with respect to the known, opposing roles of the Wnt and BMP pathways in hair regeneration. Combined with our previous study showing a common genetic battery marks hair follicle stem cells and naïve successional dental epithelia (SDE)42, these data support a model where teeth and hair, as epithelial appendages, share evolutionarily conserved genetic instructions used to regulate whole organ regeneration. Future and ongoing work will detail the genetic responses to these overexpression treatments to ask whether the genes responding to these different OE conditions also show overlap with hair regeneration.

**Regeneration rate and total tooth number can be influenced separately or in concert**

Zebrafish typically end primary expansion of their tooth fields at around 30 days old, forming a consistent arrangement of tooth families that is maintained into adulthood62. In all three of our zebrafish OE experiments, the replacement rate changed significantly, but the number of tooth families was not significantly altered. Thus, the changes in zebrafish total tooth number we observed (which includes tooth germs) were likely reflecting the higher (wnt10a) or lower (bmp6) number of tooth families that were actively undergoing regeneration or slowing shedding, causing there to be two ossified teeth at a different number of tooth positions. Conversely, zebrafish grem2a OE increased the replacement rate by both increasing new teeth and decreasing retained teeth, without changing the number of tooth families or significantly increasing total tooth number. Overall, these data suggest that zebrafish, like sticklebacks, are capable of measurably adjusting tooth replacement rates without demonstrating significant changes to tooth family number or total tooth number.
Secreted signals that simply promote or inhibit the differentiation of all tooth germs – replacement and primary – would be instead predicted to change the replacement rate while also inhibiting any normal increases to total tooth number in concert. We found that Dkk2 OE in stickleback best fit the predicted phenotype of a secreted protein that inhibits all tooth formation: new teeth, the replacement rate, and total teeth all dropped under Dkk2 OE, while retained teeth rose, indicating that tooth turnover and primary growth were both precipitously slowed (Fig. 4A). Notably, Dkk2 OE appears to have allowed bell stage tooth germs that were present at the time of OE onset to finish development (since zero early, mid- or late bell stage tooth germs were observed in OE fish). Conversely, no zebrafish treatment altered the number of tooth families visible, thus no treatment we performed here was able to affect primary tooth field expansion or contraction in adult zebrafish.

*Bmp6* and *Grem2a* OE in sticklebacks both caused a drop in retained tooth number that was not accompanied by an increase in new tooth number. These results strongly suggest that tooth shedding events can be promoted in the absence of increased new tooth formation in sticklebacks. Particularly in the *Bmp6* treatment, tooth shedding increased in the face of a sharp reduction in new teeth. Thus, in sticklebacks, the process of tooth replacement can apparently be regulated not just by influencing the start of replacement tooth formation, but also by independently regulating tooth shedding. These data further support the hypothesis that the documented evolutionary changes to tooth number and replacement rate could manifest by changes to tooth shedding mechanics in addition to the tooth formation process.

**Other differences and similarities between sticklebacks and zebrafish**

Each of our OE treatments affected some aspect of tooth regeneration in a direction predicted by a hair regeneration model. However, the specific nature of each response was not always the same between sticklebacks and zebrafish for the three gene ortholog pairs we tested in both species (*Wnt10*, *Bmp6*, and *Grem2a*). Importantly, the lack of an effect in one species vs the
other could simply reflect limitations of our OE assay: for example, the genomic integration of
the OE construct in one species could feasibly be at a genomic location that allows for stronger
or weaker transgene expression upon heat shock, causing us to observe unique effects. It is
also possible that species-specific protein sequence differences contribute to the differences we
observed. Despite these limitations of our approach, we are still able to deduce important
similarities and likely differences in how these two animals respond to the overexpression of
Wnt and BMP pathway members.

In both species, new teeth were increased by Wnt10a OE and decreased by Bmp6 OE,
suggesting that each of these secreted factors exert conserved effects between species that are
opposite in their influence on the initiation of replacement tooth growth. Grem2a, while it did
work to increase the new:retained ratio in both species, achieved this by unique means: in
sticklebacks, by only reducing the number of retained teeth (Fig. 7A), and in zebrafish by
simultaneously reducing the number of retained teeth and increasing the number of new teeth
(Fig. 7B). Grem2a thus may exert some effects in zebrafish that are not realized in sticklebacks,
but it remains possible that this secreted factor is activated by shared machinery in hair and
teeth, generally facilitating organ replacement.

In zebrafish, significant changes in the tooth replacement rate (as measured by the
new:retained ratio) were always at least partly driven by corresponding changes to the number
of new teeth. Furthermore, zebrafish retained tooth numbers showed either no detectable
change (wnt10a and bmp6) or changed in the opposite direction as new teeth (grem2a).
Sticklebacks, on the other hand, showed changes in the replacement rate that were driven
either just by changes in retained tooth number (Grem2a; Fig. 7A) or where both retained teeth
and new teeth changed in the same direction (both decreased under Bmp6; Fig. 6A). Thus,
sticklebacks twice exhibited the ability to change retained tooth number in a manner that was
unlikely a response to the increased generation of new teeth (because new teeth did not
increase), whereas zebrafish never demonstrated this phenomenon. These differences in
response between sticklebacks and zebrafish could be partly due to the differences in
regeneration strategy exhibited by these fish species: zebrafish adults maintain a set number
(11) of stationary tooth families per tooth field that undergo one-for-one replacement in
morphologically separated cycles\textsuperscript{62}, whereas sticklebacks have hundreds of teeth that do not
retain a consistent arrangement into adulthood and appear to occasionally engage in one-for-
two tooth replacement events\textsuperscript{42}. We speculate that the highly canalized tooth regeneration
process in zebrafish might contribute to there being less flexibility in the timing of tooth shedding
during replacement tooth growth than in a species like stickleback.

\textbf{Methods}

\textbf{Overexpression transgene constructs}

We used restriction-ligation cloning per standard methods to create the heat-shock
overexpression construct plasmid backbone used in all seven OE treatments described here.
We digested the pT2He-eGFP plasmid with SfiI and BglII, discarded the smaller insert, and
ligated the annealed oligos
AGGCCCCTAAAGGACTAGTCATATGTCTAGACTCGAGCGCTAGGCGCGCCGGATCCA and
GATCTGGATCCGGCGCGCCCCTAGGCTCGAGTCTAGACATATGACTAGTCCTTAGGGGCC
TATC onto the ends. This created a plasmid with a multiple cloning site between the forward
and reverse Tol2 transposase recognition sequences, including Ascl, Aval, AvrII, BamHI, BglII
DraII, SpeI, XbaI, and XhoI restriction enzyme cut sites. We named this intermediate plasmid
“T2Rv10.” Next, we added the SV40 poly adenylation signal by amplifying it from the pT2He-
eGFP reporter construct with the primers GCCGAGATCTCGATGATCCAGACATGATAAG and
GTTGTTGAATTCGCCATACCACATTTGTAGAG and ligated into T2Rv10 via restriction cloning
with BglII and EcoRI. Thereafter we used the primers
ATAGGCCAGATAGGCTCAGGGGTGTCGCTTGG and AATTGACTAGTCTTGTACAGCTCGTCCATGC to amplify the zebrafish hsp70l promoter and the mCherry coding sequence (without the stop codon) in tandem from the pT2He-mCherry reporter construct and ligated the product into the plasmid via the SfiI and SpeI restriction sites. Finally, we used the primers AATTGACTAGTGCAGCGGACC and GCCGTCTAGAGGGTCCGGGGTTCTCTTC to amplify the Porcine 2A (P2A) self-cleaving peptide coding sequence from the pMS48 plasmid and ligated it into the plasmid via restriction cloning with SpeI and XbaI, yielding the “pT2overCherry” construct used in this work. This plasmid was thereafter outfitted with coding sequences of interest (with a stop codon) downstream of the P2A coding region by using standard restriction site cloning with any two of the Ascl, AvrII, BamHI, BglIII, XbaI, or XhoI recognition sites that remain available in the multiple cloning site.

Seven coding regions for gene overexpression were synthesized by Gene Universal (Delaware, USA): stickleback Wnt10a, Dkk2, Bmp6, and Grem2a, and zebrafish wnt10a, bmp6, and gem2a (see Table S2 for accession numbers and full DNA sequences). These products were synthesized and cloned into pBlueScript SkII+ by Gene Universal, using XbaI and XhoI restriction enzyme recognition sites in all cases save stickleback Wnt10a, for which XbaI and BamHI were used due to an internal cut site for XhoI. Upon receiving these synthesized products, we digested the plasmids using the same restriction enzyme pair that was used to place them into pBlueScript and ligated them into pT2overCherry (digested with the corresponding restriction enzymes and the small insert removed). Ligation products were transformed per standard methods. Colony PCR screening was then performed to identify colonies carrying likely successful ligation products. These were miniprepped (Qiagen) and their full inserts were Sanger sequence verified, leaving ~1 mL of bacterial culture at 4° C to later inoculate a larger liquid culture for midiprep. Once verified, midipreps (Qiagen),
phenol:chloroform extractions, and DNA precipitation was performed per standard methods to prepare plasmids for injection.

**Fish husbandry and transgenic line establishment**

Zebrafish and stickleback broodstock were raised and maintained under standard conditions. Transgenesis was accomplished by injecting Tol2 mRNA and pT2overCherry plasmids containing the aforementioned coding regions (see Table S2). F0 injected fish were outcrossed to WTs, founders were identified, and a single F1 offspring was thereafter used to establish a stable line from each founder. If the F2 generation exhibited significantly more than 50% transgenic offspring, we outcrossed the line to WT until we observed ~50% transgenic offspring (eliminating insertions until we inferred there was only a single insertion). The work presented here makes use of a single insertion of each transgene for each OE treatment.

**Heat shock treatments and pulse-chase bone labeling**

To initiate an OE experiment, groups of ~15-20 sibling fish were selected for treatment based on transgene carrier status. We inferred transgene carrier status by lightly sedating fish in 50 mg/mL MS-222 and briefly observing their lenses, where the zebrafish hsp70l heat shock promoter drives sustained expression even in the absence of heat shock. WT and OE sibling fish we analyzed were always raised together in a common tank for their entire lives, and only separated briefly from each other during this sorting process. To initiate the treatment, groups of fish were placed into a tank containing a previously described86 Alizarin Red live-staining solution (0.1 g/L Alizarin Red S with 1mM HEPES) made using either stickleback or zebrafish tank/system water for each species. Sticklebacks were pulsed with Alizarin Red for 24-30 hours in 2L of solution, zebrafish were pulsed for 48-54 hours in 1L of solution (we note that 24 hours in Alizarin Red is insufficient to consistently stain retained teeth 18 days later in zebrafish). Sticklebacks were withheld from food during the Alizarin Red pulse, while zebrafish were fed a
small amount of flake food on the morning of the 2nd day while still in the Alizarin Red solution.

After the Alizarin Red staining pulse was completed, the fish were rinsed once then washed 3 times for 10-30 minutes each in fresh tank/system water before being placed into the heat shock tank. Stickleback heat shock tanks were 4L in volume, were lightly aerated with an air stone for increased water agitation (to more uniformly distribute the temperature throughout the tank) and featured two 50 watt aquarium heaters set to 29° Celsius controlled by a timer. The timer engaged the heaters twice per day for two hours per pulse, starting every 12 hours. Since it takes the 4L tanks approximately 50 minutes to ramp up to the heat shock temperature, the two hours of applied heat translates to about 70 minutes at the heat shock temperature per heat shock. Water changes were performed as needed every ~5 days, avoiding the heat shock intervals. Stickleback negative control assays were carried out in exactly the same manner, except the heaters were removed from the 4L “treatment” tank. Zebrafish heat shock tanks essentially followed a published protocol, with some modification. Standard 2.8L Aquaneering tanks with a single 50W tank heater set to 39° C were kept on either a dripping water flow rate or with no flow, and were not aerated (normal zebrafish movement was sufficient to agitate the water and create a uniform temperature throughout the tank). The timer activated the heater twice per day for 90 minutes, starting every 12 hours. Since it takes ~2.8L of zebrafish system water about 20 minutes to heat to 39° C, these treatments brought fish to the heat shock temperature for around 70 minutes per treatment, as in the stickleback heat shock treatments. Water changes were performed at least once every four days by turning the tank flow on high for 10+ minutes, again avoiding the heat shock intervals. After the 36th heat shock on the 18th day of the treatment, both species of fish were withheld from feeding, removed from their respective heat shock tanks, and live stained in a calcien live staining solution (0.05 g/L calccein with 1mM sodium phosphate) for 16-18 hours, in 2L for sticklebacks and 1L for zebrafish. After rinsing and washing the fish in their corresponding tank/system water at least
three times over 30 min, fish were euthanized in 250 mg/mL MS-222, sorted by red channel fluorescence, and fixed in 4% PFA overnight at 4° C with high agitation.

Preparation and blinding of pulse-chase labeled tooth fields

Following fixation, fish from OE experiments were rinsed and washed in tap water, then agitated in 1% KOH for 20+ minutes at room temperature. Dental tissues were dissected out in system water or 1X PBS, then washed at room temperature with 1% KOH overnight or with 5% KOH for 30-60 minutes, rendering the mCherry signal no longer detectable. Dental tissues were then washed through a glycerol series (25, 50, 90% glycerol in 1x PBS). Stickleback pharyngeal tissues were flat mounted as previously described87, while zebrafish pharyngeal tooth fields and stickleback oral jaws were arrayed into 24 well plates. The resulting pulse-chase dental samples were then shuffled and renamed irrespective of treatment condition so that a different researcher could blindly score and count the teeth in each dental preparation (i.e. the researcher scoring the teeth did not prepare the dissected tooth fields nor did they know which individuals carried an OE transgene).

Scoring and analyzing pulse-chase assays

Dental preparations were scored on a Leica M165 stereomicroscope using GFP2, strict GFP, and Rhodamine filters to observe Alizarin Red and calcein staining. Every tooth in each animal was addressed for both species (left and right premaxillary, dentary, DTP1, DTP2, and VTP tooth fields in sticklebacks, and the VTP in zebrafish). Importantly, we find that the pulse-chase signal is markedly more difficult to interpret after ~1 week at room temperature or ~3 weeks stored at 4° C, both because the calcein signal fades and because autofluorescence in soft tissues increases. Skeletal preparations from a given experiment were thus always scored within two days of the end of each experiment, and always within a single 24 hour window. Since actively growing bony tooth tissues (dentine and enameloid) strongly incorporate these
stains, this pulse-chase strategy allows us to classify each tooth in each individual as either 
“new” or “retained” with respect to the treatment interval: “new” teeth are those that began bone 
deposition after the Alizarin Red pulse (during the OE treatment), and are thus only marked by 
the 2nd stain, calcein, while “retained” teeth are those that show any Alizarin Red stain, because 
this indicates that these teeth were depositing bone prior to the treatment interval (see Fig. 2F-H). The only exception to these rules was in the stickleback Bmp6 OE treatment, where tooth 
germs in the treatment condition were observed without either stain. Because these tooth germs 
were Alizarin Red negative, we inferred that these were new teeth despite their lack of calcein 
stain; i.e. we interpret that this class of tooth germs had halted bone deposition by the time the 
calcein chase occurred. The new:retained ratio and total tooth number for each fish was 
additionally calculated by dividing or summing new and retained counts, respectively. We 
additionally counted morphological tooth families in each zebrafish specimen to address primary 
tooth number expansion or contraction in this species. All statistical tests were performed in R, 
using two-sided Wilcoxon rank-sum tests in all cases. After scoring was complete, example 
pulse-chase sample examples were imaged on a Leica DM2500 compound microscope, Leica 
M165 stereomicroscope, or Zeiss LSM 700 confocal microscope.

In situ hybridization

In situ hybridization on sections from subadult and adult sticklebacks was performed as 
previously described42. See Table S1 for probe template sequence information. Probes 
designed to Wnt10a and Bmp6 were published previously42,54. A 3’ UTR probe template for 
Grem2a was cloned from genomic DNA via PCR using the primers 
GGTGCAGAGGGTCAAACAGT and ATACAGGCTCGTGTCCAAGC. The probe template for 
Dkk2 was created from the purchased full-length coding sequence (Gene Universal, Delaware, 
USA) that was also used to create the overexpression construct (described above). 
Digoxigenin-labeled in situ riboprobes were synthesized as previously described42. WT material
was embedded in paraffin and sectioned as previously described on either the sagittal or coronal plane\textsuperscript{42}.

**Hematoxylin and Eosin staining**

\textit{Dkk2} OE and sibling WT fish underwent a typical OE treatment but were not pulsed or chased with Alizarin Red or calcein. These fish were then fixed and sorted as described above. Thereafter these fish were decapitated, and their heads were prepared for sectioning on the coronal plane a previously described\textsuperscript{42}. Sections were stained with Hematoxylin and Eosin as previously described\textsuperscript{42}.

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**Figure legends**

**Figure 1.** \textit{In situ} hybridization detects dynamic expression of \textit{Wnt10a}, \textit{Dkk2}, \textit{Bmp6}, and \textit{Grem2a} within and surrounding pharyngeal tooth organs in subadult wild-type sticklebacks. The basalmost layer of epithelium is flanked by black dotted lines in each image. Arrows mark dental epithelium, arrowheads mark dental mesenchyme, black pointers mark detected expression, white markers indicate regions with no detected expression, brackets mark mesenchymal expression outside of tooth organs, and carets mark the successional dental epithelium. **A-D.** \textit{Wnt10a} transcripts were detected focally in bud stage tooth germs (A), in both epithelium (black arrow) and mesenchyme (black arrowhead). In mid-bell stage tooth germs (B), expression was detected in the inner dental epithelium (black arrow) and tooth mesenchyme (black arrowhead).
In late bell (D) and eruption stage teeth (C), epithelial expression was no longer detected (white arrows), but mesenchymal expression persists (black arrowheads). In fully ankylosed and erupted teeth (C and D), *Wnt10a* expression was not widely detected. *Wnt10a* expression was additionally detected in localized mesenchymal cells surrounding tooth organs (bracket in A). E and F. *Dkk2* transcripts diffusely mark pharyngeal dental epithelium (upper portion of both images), including early bud stage tooth germs (left black arrow in E), but are not detected in bud stage mesenchyme (white arrowhead in E). Epithelial transcripts were most strongly detected in mid-bell stage inner dental epithelium (right black arrow in E), and mid-bell mesenchyme (black arrowhead in E). Expression is additionally detected in mesenchymal clusters near the bone of attachment (bracket in E). Expression in tooth mesenchyme is additionally detected in fully ankylosed erupted teeth (black arrowhead in F). G-J. *Bmp6* transcripts were detected in bud stage tooth epithelium and mesenchyme (black arrow and arrowhead in G), bell stage inner dental epithelium and mesenchyme (black arrow and arrowhead in H). *Bmp6* was not detected in eruption stage tooth epithelium (white arrow in I) but was detected in eruption stage mesenchyme (black arrowhead in I) and erupted tooth mesenchyme (black arrowhead in J). *Bmp6* was also transcribed in some successional dental epithelia (caret in I). Localized mesenchymal expression between teeth was also detected (brackets in I). K-M. *Grem2a* transcripts were not detected in bud stage tooth germ epithelium or mesenchyme (white arrow and arrowhead in K). Bell stage tooth germs and eruption stage teeth exhibited expression in inner and outer dental epithelium (black arrows in L and M). Tooth mesenchyme was *Grem2a* positive in bell stages through fully ankylosed, erupted teeth (black arrowheads in L and M). *Grem2a* was additionally detected in mesenchyme surrounding tooth organs (brackets in K and M) and in the successional dental epithelium (caret in M). Scale bars: 20 μm.
**Figure 2.** Summary of the two-stain pulse-chase heat shock method for assessing tooth gain and loss. **A.** A schematic of the transgene used. The zebrafish *hsp70l* promoter drives the expression of an mCherry-P2A-CDS transgene, where “CDS” is one of seven full length coding sequences of interest (see Methods and Table S2). P2A (Porcine 2A) is a 22 amino acid sequence that has been shown to self-cleave on translation between residues 20 and 21. This separates mCherry from the CDS, leaving the bulk of the P2A protein on the C terminus of mCherry. **B.** Example temperature profiles for heat shock treatments. Alizarin pulse and calcein chase are shown as pink and green bars, respectively. **C.** Lateral images of sticklebacks 24 hours after a single heat shock. Control (WT) on top, overexpression transgene positive (OE) fish on bottom (*Bmp6* OE in this example). C’ shows red channel fluorescence, allowing us to visualize the transcription of our overexpression transgene. **D and E.** Dissecting the ventral tooth plate (VTP) from each WT and OE fish reveals mCherry localized to the tooth field itself (E’ shows red channel fluorescence). Anterior to top. **F.** Example images of the pulse-chase method on a control individual. Alizarin Red (F) strongly marks all bone undergoing active ossification at the start of the treatment (false colored pink). F’. 18 days later, after 36 heat shocks, a calcein chase marks all bone ossifying at the end of the treatment (green). F”.

Overlain images of stickleback pharyngeal teeth showing Alizarin Red and calcein reveals whether individual teeth were either present at only the 2nd labeling (new, calcein only, example marked “n”) or if they were present at both the first and the second labeling (retained, Alizarin Red and calcein positive, example marked “r”). **G.** An overlay of the pulse-chase treatment on zebrafish teeth using the same treatment interval (see panel B, zebrafish example).

**Figure 3.** Effects of *Wnt10a* overexpression in stickleback and zebrafish. **A.** In sticklebacks, the number of new teeth (*P*=0.00072), retained teeth (*P*=0.010), and the new:retained ratio (*P*=0.0003) showed significant differences between WT and OE fish, however the total number of teeth did not (*P*=0.81). **B and C.** Overlay images of stickleback ventral tooth plates showing
Alizarin Red and calcein signal in control (B) and OE (C) individuals. Note clusters of new teeth (white dotted circle showing an example on the left VTP). D. In zebrafish, significant increases in the number of new teeth ($P=0.00039$), the new:retained ratio ($P=0.0042$), and total teeth ($P=0.011$) were found but retained tooth number ($P=0.56$) did not significantly change.

**Figure 4.** Effects of *Dkk2* overexpression in stickleback. A. Significant decreases in the number of new teeth ($P=0.00024$), the new:retained ratio ($P=1.1e-5$), and total teeth ($P=0.0036$) were detected, while an increase in retained teeth ($P=0.045$) was observed. B and C. Overlay images of stickleback ventral tooth plates showing Alizarin Red and calcein signal in control (B) and OE (C) individuals. Note near complete absence of unankylosed new teeth in the OE individual (white arrow in C) compared to the WT individual (white arrows in B). The right side is left unlabeled. D and E. Hematoxylin and Eosin (H&E) staining reveals no tooth germs of any stage in *Dkk2* OE fish (n=7/7), suggesting that this treatment does not cause tooth germs to arrest (yellow ovals in D show tooth germs, asterisks in E sit above positions normally populated by tooth germs).

**Figure 5.** Stalled tooth germs resulting from the Bmp6 overexpression treatment. A shows WT control, B shows OE transgene carrier. A and B show brightfield, A’ and B’ show green channel fluorescence (calcein), A” and B” show an overlay. Note that there is no caclein signal in the four tooth germs indicated in the Bmp6 OE individual (gray arrows in B’). Black and white arrows otherwise mark tooth germs in those panels where they are visible.

**Figure 6.** Effects of Bmp6 overexpression in stickleback and zebrafish. A. In sticklebacks, significant decreases in all four variables were detected: the number of new teeth ($P=2.7e-5$), the number of retained teeth ($P=2.8e-5$), the new:retained ratio ($P=4.4e-5$), and total teeth ($P=2.8e-5$). B and C. Overlay images of stickleback ventral tooth plates showing Alizarin Red and calcein signal in control (B) and OE (C) individuals. Note regions usually populated with ankylosed teeth are completely devoid of any such structure (dotted oval in D, the right side is
D. In zebrafish, significant decreases in the number of new teeth \((P=0.0042)\), the new:retained ratio \((P=0.013)\), and total teeth \((P=0.0035)\) were detected but retained tooth number \((P=0.78)\) did not significantly change.

**Figure 7.** Effects of Grem2a overexpression in stickleback and zebrafish. **A.** In sticklebacks, significant decrease in the number of retained teeth \((P=0.039)\), a significant increase in the new:retained ratio \((P=0.029)\), but no significant changes to new teeth \((P=0.64)\) or total teeth \((P=0.15)\) were observed. **B.** In zebrafish, significant increases in the number of new teeth \((P=0.00031)\), and the new:retained ratio \((P=0.00013)\) were found, and a significant decrease in retained teeth \((P=0.0047)\), however no significant change in total teeth \((P=0.14)\) was observed. **C and D.** Overlay images of zebrafish ventral tooth plates showing Alizarin Red and calcein signal in control (B) and OE (C) individuals. White circles indicate new teeth, solid circles mark those that are superficial, dotted circles mark those that are deep in the tooth field and thus occluded by erupted teeth.

**Figure S1.** Results of stickleback *Wnt10a* OE parsed by tooth field. Stickleback pharyngeal teeth are housed in three discrete fields: the 1st dorsal tooth plate 1 (DTP1), the 2nd dorsal tooth plate (DTP2), and the ventral tooth plate (VTP). In the oral jaws, sticklebacks have teeth on their premaxilla (Premax) and dentary (Dent) bones. Each group of graphs shows new, retained, the new:retained ratio, and total tooth number broken down by tooth field type (the sum of left and right halves per fish).

**Figure S2.** Negative control experiment with *Wnt10a* transgene carriers. The same pulse-chase interval (18 days, see Methods) with no heat shocks was applied to full siblings of the individuals in the experiment summarized in Fig. 3A, testing for whether simply carrying the transgene in the absence of any heat shock is associated with changes in the number of new teeth, retained teeth, the new:retained ratio, or total teeth; no such deviations were detected in this negative control experiment for any of these four variables assessed (all \(P>0.68)\).
**Figure S3.** Results of stickleback 
*Dkk2* OE parsed by tooth field. Stickleback pharyngeal teeth are housed in three discrete fields: the 1st dorsal tooth plate 1 (DTP1), the 2nd dorsal tooth plate (DTP2), and the ventral tooth plate (VTP). In the oral jaws, sticklebacks have teeth on their premaxilla (Premax) and dentary (Dent) bones. Each group of graphs shows new, retained, the new:retained ratio, and total tooth number broken down by tooth field type (the sum of left and right halves per fish).

**Figure S4.** Results of stickleback Bmp6 OE parsed by tooth field. Stickleback pharyngeal teeth are housed in three discrete fields: the 1st dorsal tooth plate 1 (DTP1), the 2nd dorsal tooth plate (DTP2), and the ventral tooth plate (VTP). In the oral jaws, sticklebacks have teeth on their premaxilla (Premax) and dentary (Dent) bones. Each group of graphs shows new, retained, the new:retained ratio, and total tooth number broken down by tooth field type (the sum of left and right halves per fish).

**Figure S5.** Negative control experiment with 
*Bmp6* transgene carriers. The same pulse-chase interval (18 days, see Methods) with no heat shocks was applied to full siblings of the individuals in the experiment summarized in Fig. 6A, testing for whether simply carrying the transgene in the absence of any heat shock is associated with changes in the number of new teeth, retained teeth, the new:retained ratio, or total teeth; no such deviations were detected in this negative control experiment for any of these four variables assessed (all *P*>0.26). Additionally, no “stalled” tooth germs were observed.

**Figure S6.** Results of stickleback Bmp6 OE parsed by tooth field. Stickleback pharyngeal teeth are housed in three discrete fields: the 1st dorsal tooth plate 1 (DTP1), the 2nd dorsal tooth plate (DTP2), and the ventral tooth plate (VTP). In the oral jaws, sticklebacks have teeth on their premaxilla (Premax) and dentary (Dent) bones. Each group of graphs shows new, retained, the new:retained ratio, and total tooth number broken down by tooth field type (the sum of left and right halves per fish).
References cited


A. Stickleback Wnt10a overexpression

- **New teeth**
  - WT: [Boxplot]
  - OE: [Boxplot]
- **Retained teeth**
  - WT: [Boxplot]
  - OE: [Boxplot]
- **New:retained ratio**
  - WT: [Boxplot]
  - OE: [Boxplot]
- **Total teeth**
  - WT: [Boxplot]
  - OE: [Boxplot]

B. Images showing new and retained teeth in sticklebacks under WT and OE conditions.

C. Images showing new and retained teeth in sticklebacks under WT and OE conditions.

D. Zebrafish wnt10a overexpression

- **New teeth**
  - WT: [Boxplot]
  - OE: [Boxplot]
- **Retained teeth**
  - WT: [Boxplot]
  - OE: [Boxplot]
- **New:retained ratio**
  - WT: [Boxplot]
  - OE: [Boxplot]
- **Total teeth**
  - WT: [Boxplot]
  - OE: [Boxplot]
A

Stickleback Dkk2 overexpression

New teeth

Retained teeth

New:retained ratio

Total teeth

WT  OE

B

C

D

E

100 μm

Legend:

WT = Wild Type
OE = Overexpression

Statistical significance:

*** p < 0.001

Imaging:

B

C

D

E

Legend:

D: Hematoxylin and Eosin staining
E: Immunofluorescence staining

Arrows indicate specific regions of interest.

Scale bar: 100 μm
A. **Stickleback Bmp6 overexpression**

- **New teeth**
  - WT: 120, OE: 160
  - WT: 100, OE: 140
- **Retained teeth**
  - WT: 120, OE: 160
  - WT: 100, OE: 140
- **New:retained ratio**
  - WT: 1.1, OE: 1.1
  - WT: 0.8, OE: 0.8
- **Total teeth**
  - WT: 280, OE: 280
  - WT: 260, OE: 260

B. **Zebras**

C. **Bmp6 overexpression**

D. **Zebrafish Bmp6 overexpression**

- **New teeth**
  - WT: 5, OE: 10
  - WT: 6, OE: 8
- **Retained teeth**
  - WT: 22, OE: n.s.
  - WT: 18, OE: 18
- **New:retained ratio**
  - WT: 0.8, OE: 0.8
  - WT: 0.6, OE: 0.6
- **Total teeth**
  - WT: 28, OE: 30
  - WT: 26, OE: 28
Stickleback Bmp6 negative control

- **New teeth**
- **Retained**
- **New:retained ratio**
- **Total teeth**

Graphs showing data for new teeth, retained teeth, new:retained ratio, and total teeth across different transgene conditions.