Control elements targeting Tgfb3 expression to the palatal epithelium are located intergenically and in introns of the upstream Ift43 gene

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INTRODUCTION

Failure of palatogenesis (palate formation) results in cleft palate, which is one of the most common congenital birth defects in humans. In mice, palatogenesis starts around embryonic day 11.5 (E11.5) when bilateral outgrowths of the maxillary process called palatal shelves start to grow down vertically on each side of the tongue. Co-ordinated growth of palatal shelves themselves, the tongue and the rest of the oral cavity is followed by rapid palatal shelf elevation (∼E14) and fusion (∼E15) (Bush and Jiang, 2012).

Palatal shelves are composed of the neural crest-derived mesenchyme covered by epithelial cells (Bush and Jiang, 2012). Before palatal fusion, the epithelial layer is composed of a basal layer of cuboidal medial edge epithelial (MEE) cells and an apical peridermal layer of flattened cells. This peridermal layer is shed from the tips of the apposed elevated palatal shelves just before they form contact with one other, allowing adhesion and intercalation of the underlying MEE cells (Yoshida et al., 2012) to form a midline epithelial seam. Epithelial cells in this seam are subsequently lost and the underlying basement membrane degraded resulting in palatal mesenchymal confluence (Gritli-Linde, 2007).

Several studies have demonstrated that signaling triggered by transforming growth factor-beta (TGF-β) plays a critical role in palatal epithelial fusion. Tgfb3 is strongly and specifically expressed in MEE cells (Fitzpatrick et al., 1990; Pelton et al., 1990; Millan et al., 1991), and mice lacking Tgfb3 display 100% penetrant cleft secondary palate (Kaartinen et al., 1995; Proetzel et al., 1995), which results from defects in TGF-β3-induced palatal MEE differentiation and/or apoptosis (Kaartinen et al., 1997; Taya et al., 1999; Ahmed et al., 2007; Iwata et al., 2013). Results of a recent study also suggest that TGF-β3 is required for peridermal desquamation (Wu et al., 2013). Mutations in the human TGFB3 have been linked to cleft palate (Lidral et al., 1998; Carinci et al., 2007), and a recent report described a disease-causing mutation in the coding region of TGFB3 in patients showing abnormalities in palate and muscle development (Rienhoff et al., 2013).

A commonly used approach to study complex developmental processes has been to manipulate gene function in mouse models using the Cre-lox system (Rajewsky et al., 1996). In the context of palatogenesis, an epithelium-specific keratin14-Cre (K14-Cre) driver line (Andl et al., 2004) has been frequently used, since it recombines with a very high efficiency in the MEE (Dudas et al., 2006; Xu et al., 2006). Yet abrogation of the Tgfbr1 gene encoding the TGF-β type I receptor (Dudas et al., 2006) or Tgfb3 in the palatal epithelium (this study) resulted in a significantly milder palatal phenotype than systemic deletion of the Tgfb3 gene encoding the TGF-β ligand (Kaartinen et al., 1995; Proetzel et al., 1995). Here we show that this phenotypic difference is likely caused by an inability of the K14-Cre driver to recombine in peridermal cells.
To better understand how gene expression is specifically directed in the pre-fusion MEE and overlying peridermal cells, we decided to identify control elements responsible for palate-specific Tgfb3 expression. We surveyed more than 400 kilobases (kb) of mouse genomic DNA sequences on mouse chromosome 12, and identified a 61-kb fragment around the Tgfb3 gene that directs reporter expression specifically in the MEE and adjacent periderm. Using transient transgenic approaches, we identified three smaller cis-regulatory regions: one in the proximal intergenic region and two in intron 2 of the upstream gene. These more distal elements may function as “shadow” enhancers assuring robust and reliable control of Tgfb3 expression in the MEE and adjacent periderm.

EXPERIMENTAL PROCEDURES

ANIMAL CARE

This study was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All the experiments involving animals described in this study were approved by the Animal Care and Use Committee of the University of Michigan-Ann Arbor (protocol number: PRO00004320).

BACs and BAC recombineering

Mouse BACs RP23-76M13 (=5′BAC) and RP24-299H18 (=3′BAC) were obtained from Children's Hospital Oakland Research Institute (http://bacpac.chori.org) (see Figure 2A). Their identity was verified using a standard restriction mapping technique (data not shown).

Insertion of the SA-lacZ-PA cassette into exon1 of the 5′ BAC RP23-76M13 and the 3′ BAC RP24-299H18 (see Figure 2A)

Targeting arms were generated by PCR using BAC RP23-76M13 as a template and the following primers:

- Tgfb3-L1: 5′-TCCTAGCTCTACCACGGACACAG-3′
- Tgfb3H3xH1-L2: 5′-AAGGGTCTCTGAGTGTGAGGCCAGGAA CGAG-3′
- Tgfb3H3xH1-R1: 5′-CTCAGAGAAGTCTGAAGGCTCTGGTA GTCTCTG-3′
- Tgfb3R2: 5′-TGATAGGGGACGTGGGTCATC-3′

pNASSβ (SA-lacZ-PA) cassette was inserted into exon 1 of the BACs RP23-76M13 and RP24-299H18 using standard BAC recombineering techniques (Warming et al., 2005). Neo 452 (a loxP-Neo-PA-loxP cassette) was added to the generated BAC to enable selection with kanamycin. Integrity of the recombinered BACs was confirmed by PCR after amplification.

Preparation of the 61-kb and 28-kb BACs

The 61-kb BAC: A 128-kb 3′ fragment from the recombinered BAC RP24-299H18 was deleted in two steps. First, a targeting vector to replace the large 3′ fragment with pGalK was generated by using the primers:

- 3′del-F: 5′-TGACAGATATAGGCGCTGTTGAGAACTCGCCATT AGCAGAGAGGCGCGCTGTAAGAGGCTGGCCCTTCTGAA TTCTCACC-3′

After successful recombinering, a targeting vector to delete the pGalK selection marker was generated by PCR using the following primers:

- 3′del-pGalK-F: 5′-AGAATCTGCGCATTGAGGCCGAGGCGCA TCGTTGCCCTTCTGAATTCAAAAGTCTAATACCTT CCTACCCCTGCGGGAAAGTAAATGTCGCTAAACAC-3′
- 3′del-pGalK-R: 5′-GTTTTTGGACACTACCTTTTCCCCAGG GTCAGGAACTGTAGACCTTTGTAGAATTCGAGGG GGCAGGATGGGCGCTCCCGCTAATTGCGAGTTCT-3′

The targeting vector was deleted as described (Warming et al., 2005).

The 28 kb BAC: A 33-kb 5′ fragment was deleted from the 5′ end of the 61-kb BAC as outlined above. Primers to generate the targeting vector were:

- 5′3′del-F: 5′-TGACAGAGGAGGCGGCTGTTGATAGATGACT AATGTCATCCTGCCA-3′
- 5′3′del-R: 5′-GCTGCTCTACCGCAGGAACCCGTGC TGATTCTCTGTTAGAATTCGAGGG GGCAGGATGGGCGCTCCCGCTAAT GTGCGCTCCT-3′

The targeting vector to delete the pGalK selection marker was generated by PCR and the following primers:

- 5′3′del-pGalK-F: 5′-AGGGCTGTATGAGTACTCGGCA CCTGATCGTATGAGGAACACGATCTTCTCTGTTGAC AATGTTATTCAAC-3′
- 5′3′del-pGalK-R: 5′-GATGTCATCAACGGGCTGCTGCTT AATGCAATGATCCAAACAGGGGCTGCC TAACTCGGAAAGGATTATTTTAACCTGTATACGAC CGTTCTCCT-3′

BAC DNAs were purified for microinjections using Nucleobond AX alkaline lysis protocol according to the manufacturer’s instructions (Clontech).

PREPARATION OF SMALLER REPORTER CONSTRUCTS

The 2xHS4-hsp68-lacZ-PA-2xHS4 vector was generated by replacing a SacII-SacI fragment from the pUbC-SH-Gm-4xHS plasmid (kindly provided by R. Behringer) with the hsp68-lacZ-PA cassette. A unique NotI site just upstream of the hsp68 minimal promoter was generated by using the Quikchange-II site-directed mutagenesis kit (Agilent). Regions of interest were PCR-amplified using SuperMix High Fidelity polymerase (Invitrogen) (primer sequences shown in Table 1), and the generated fragments inserted into the NotI site using the In-Fusion HD cloning kit (Clontech). Plasmid DNAs were purified using endonuclease-free Maxi-Prep columns (Qiagen) and the purified DNAs were linearized by Sall for microinjection.
ALIGNMENT OF ORTHOLOGOUS SEQUENCES AND IDENTIFICATION OF PUTATIVE BINDING MOTIFS
Multi-species sequence comparisons around the Tgfb3 gene were performed using the UCSC genome browser (http://genome.ucsc.edu) and VISTA tools for Comparative Genomics (http://genome.ucsc.gov/vista) using the global pair-wise and multiple alignment (LAGAN) program. The threshold used for evolutionary conservation was 70% sequence similarity within 100 bp region of DNA sequence. Predicted transcription factor binding sites were identified by using RankVISTA and TRANSFAC matrices.

GENERATION OF TRANSGENIC MOUSE LINES AND TRANSIENT TRANSGENIC MOUSE EMBRYOS
The transgenic mouse lines and transient transgenics were generated in the Transgenic Animal Model Core facility at the University of Michigan—Ann Arbor.

OTHER MOUSE LINES USED IN THIS STUDY
We generated epithelium-specific Tgfb3 mutants by crossing mice heterozygous for the floxed Tgfb3 allele (Tgfb3Flox) (Doetschman et al., 2012) and carrying the epithelial K14-Cre driver (Andl et al., 2004) with homozygous floxed Tgfb3 (Tgfb3Flox) mice. R26R-YFP reporter mice were obtained from the Jackson Laboratories, and generation of Tgfb3-Cre mice has been previously described (Yang et al., 2008).

X-GAL STAINING
To detect expression of β-galactosidase encoded by the lacZ reporter gene, embryos were collected, washed and fixed in freshly prepared 4% para-formaldehyde-0.5% glutaraldehyde for 20 min, washed 3 × 20 min in the detergent wash solution and stained from 4 h to overnight in X-Gal staining solution as described (Behringer et al., 2003). The stained samples were examined using a Leica MZ95 dissecting microscope and photographed using an Olympus DP71 camera as described above.

RESULTS
PALATAL PERIDERMAL CELLS ARE NOT RECOMBINED IN A COMMONLY USED K14-CRE MOUSE LINE
Comparison of the palatal phenotypes of global Tgfb3 knock-out mice (Tgfb3−/−) and epithelium-specific Tgfb3 (Tgfb3:K14-Cre) mice revealed that, despite the efficient recombination in the MEE, the germline mutants consistently displayed a more severe phenotype than the tissue-specific mutants (Figures 1A–I). Tgfb3−/− mice had a complete cleft of the secondary palate (Kaartinen et al., 1995; Proetzel et al., 1995), while Tgfb3:K14-Cre mice had a cleft anteriorly, but superficial or complete fusion in the mid-palate, and an aberrant posterior epithelial bridge. Since the Tgfb3:K14-Cre palatal phenotype was practically identical to that observed in the epithelium-specific TGF-β receptor mutants (both Tgfb1:K14-Cre and Tgfb2:K14-Cre) (Dudas et al., 2006; Xu et al., 2006), we wondered whether this milder palatal phenotype was caused by an inability of the K14-Cre driver line (Andl et al., 2004) to induce recombination in peridermal cells. To address this question we harvested tissues from K14-Cre, R26R-YFP reporter embryos at E13.5, and assessed the Cre-induced recombination in MEE and peridermal cells (Figures 1J,K). Our
neighboring genes are not expressed in pre-fusion palatal shelves. The 5′ flanking sequences are remarkably short (3–3.5 kb) but the 5′ region from –263 to +26 kb (defining Tgfb3 transcriptional start site (TSS) as 0) which included the Tgfb3, Ift43, and Gpatch2l genes (green) and an SSEA1-positive subset of non-recombined peridermal cells (red, white arrow). Light blue arrow points to the DAPI-positive nucleus of a peridermal cell that is SSEA1-negative and has not been recombined by 5′ of each BAC using standard recombinant lacZ reporter BACs were used to generate transgenic mice and transgenic embryos were also stained in mammary placodes, whisker follicles, nostrils, and vasculature (Figures 2B,F,154 kb which included the Tgfb3 gene and the Ttll5 (variant 4) gene (Figure 2A). The sequences of the two BACs overlapped by 61 kb, which includes all the Tgfb3 exons and some of those of the neighboring genes.

To prepare reporter constructs, we inserted an SA-lacZ-pA cassette into Tgfb3 exon 1 of each BAC using standard recombineering techniques (Warming et al., 2005). These recombinant lacZ reporter BACs were used to generate transgenic mice and β-galactosidase activity assessed at E14.0 using X-Gal staining as Tgfb3 is usually strongly expressed in palatal shelf MEE and nasal septal epithelium around this stage. Both 5′ and 3′ lacZ-RECOMBINANT REPORTER BACs were able to target reporter activity correctly to the palatal midline and nasal septal tissues (Figures 2B,F). The 5′ lacZ-BAC transgenic embryos were also stained in mammary placodes, whisker follicles, nostrils, and vasculature (Figures 2C–E), while the 3′lacZ-BAC embryos showed additional staining principally in vasculature (Figures 2G–I, 3, which illustrates the positions of all DNA fragments tested for enhancer activity in this study and summarizes expression data). Stable transgenic mouse lines carrying 3′ lacZ-BAC did not show detectable reporter activity until E12.0–E12.5 (Figure 2J), when staining was seen first in blood vessels and soon afterwards in the tips of the posterior palatal shelves (Figures 2K,L). At E14.5 strong staining occurred along the entire A-P axis of the palatal shelf tips, and continued during and after palatal epithelial fusion when it could still
FIGURE 2 | Recombinant reporter BACs used to detect Tgfb3 regulatory regions. (A) Schematic representation of Tgfb3-containing lacZ BAC clones. Exons of the Tgfb3 gene are shown as red vertical bars and exons of Tll5, Ift43, and Gpatch21 genes are shown as green, blue and purple vertical bars, respectively. Horizontal black arrows show the direction of transcription of each gene. (B–E) 5′ lacZ BAC transgenic embryos showing β-gal reporter activity (blue staining) in tips of palatal shelves (B, black arrows), nasal septum (B, asterisk, at anterior end of palate), blood vessels (C, green arrow), mammary placodes (C, red arrows) and in whisker follicles (D, blue arrows). (F–I), 3′ lacZ BAC transgenic embryos showing β-gal reporter activity (blue staining) in tips of palatal shelves (F, black arrows), nasal septum (F, asterisk), blood vessels (G, green arrows). (B,F) roof of mouth at E14, inferior view; (C,G) torso at E14, right lateral view, (D,H) head (mandible removed), left lateral view, (E,J) head (mandible removed), frontal view. (J–N) Reporter activity in 3′ lacZ-BAC transgenic embryos between embryonic days 10.5 and 16.0. Black arrows point to expression in tips of pre-fusion palatal shelves (K,L) and midline seam (N); green arrows point to positively staining blood vessels (K–N); asterisk (in M) marks the nasal septum PS, palatal shelf. (B,F,K–N), anterior, top; posterior, bottom. Scale bars in (K), 450 μm; (B,F,J,L,N), 500 μm; (C,E,G,H), 1 mm.

be detected in the degrading midline seam and in vasculature at E16.0 (Figures 2M,N).

As the 5′ and 3′ BAC sequences overlapped, and both the 5′ and 3′ lacZ-BAC reporters drove expression in the palatal shelf tips, we hypothesized that sequences in the region common to each BAC may be responsible. We tested this by making a reporter lacZ-BAC containing only this sequence, from −35 to +26 kb (Figures 3A, 4Aa). This 61-kb fragment of mouse genomic DNA consistently drove highly specific reporter expression in the MEE and the adjacent periderm, and in peridermal cells covering the nasal septum where anterior secondary palatal fusion occurs (Figures 4B,F–H). The only other tissue showing detectable though weak X-Gal staining was the lens (Figures 4D,E).

NONCODING EVOLUTIONARILY CONSERVED SEQUENCES WITHIN THE Tgfb3 GENE ARE NOT RESPONSIBLE FOR THE MEE-SPECIFIC GENE EXPRESSION

We analyzed the 61-kb overlapping region using the UCSC Genome Browser (http://genome.ucsc.edu) (Figures 4Ac–e) to identify non-coding evolutionarily conserved regions (ECRs), which are likely to include tissue-specific enhancers and found four: ECR-I (2 kb upstream of TSS), ECR-II and ECR-III (in Tgfb3 intron 1) and ECR-IV (in Tgfb3 intron 3) (Figure 5). As these are all highly conserved in placental mammals, which develop a complete secondary palate, but not in avians (or fish), which do not express Tgfb3 in tips of palatal shelves and do not develop the fused secondary palate (Sun et al., 1998), they were good candidates to regulate palate-specific expression. To test this, the ECRs (I-IV) were PCR-amplified and subcloned upstream of the minimal hsp68 promoter and lacZ-PA reporter, and the resulting ECR-(I-IV)-hsp68-lacZ-PA cassette cloned between concatamerized pairs of genomic insulators (cHS4), which have been shown to reduce positional effects of transgenes and alleviate promoter interference (Yahata et al., 2007; Griswold et al., 2011) (Figure 5). Transient transgenic embryos were generated and analyzed for reporter activity at E14.0. While the ECRs were consistently able to target the reporter activity to several tissues (teeth, whisker follicles, nostrils and
FIGURE 3 | Enhancer screening across the Tgfb3 region. (A) Schematic representation of the regions examined to locate cis-regulatory sequences directing reporter activity to the MEE/periderm cells. (a) 417-kb of genomic DNA (black line) including the Tgfb3 gene (red boxes represent exons); colored lines beneath correspond to the positions of BAC sequences used for expression regulation analysis (see B, first four columns). (b) Schematic representation of the 61-kb region in common between the 5′ and 3′ BACs (brown line) showing the Tgfb3 gene (exons in red), exons 1–3 of the Ift43 gene (blue boxes) and exon 16 of the Ttll5 gene (green box). Graph above shows evolutionary sequence conservation among placental mammals (UCSC genome browser) along this sequence. Orange pentagons indicate positions of ECRs I-VII (see main text); colored lines beneath correspond to the positions of regions used for regional expression regulation analysis (see B, fifth column onwards). (B) A table summarizing findings of reporter activity driven by the sequences in the regions indicated by colored bars in (A) in various tissues of transgenic embryos at E14. Data entries show the number of embryos displaying positive reporter activity in selected tissues (rows) over the total number of lacZ-positive embryos for each sequence (identified by a colored bar in each column); dark yellow highlight, 50% or more staining; pale yellow highlight, >0%, <50% staining. 

forebrain), no staining was seen in the MEE (n = 15) (Figure 5). We also modified the 61-kb lacZ-BAC by deleting sequences from −35 to −3 kb. This 28-kb BAC was also unable to direct lacZ reporter expression to the MEE (Figure 3). These data suggest that the sequences from −3.0 to +26 kb, including the entire Tgfb3 gene and ECRs I-IV, are not responsible for the MEE/periderm-specific gene expression in mouse embryos during palatogenesis.

CIS-REGULATORY ELEMENTS DIRECTING GENE EXPRESSION IN THE MEE ARE LOCATED IN INTRON 2 OF THE UPSTREAM Ift43 GENE

In addition to the noncoding ECRs I-IV, the 61-kb region from −35 to +26 kb contains three additional highly conserved regions in intron 2 of the Ift43 gene: ECR-V at position −(7.9 to 7.6) kb, ECR-VI at −(13.0 to 12.5) kb and ECR-VII at −(26.9 to 24.0) kb. To assess these regions for the possible presence of MEE/periderm-specific cis-regulatory elements, we
first subcloned the ECRs -V and -VI into the 2xcHS4-hsp68-lacZ-2xcHS4 vector as a single 7.6-kb fragment (Figure 5A) and ECR-VII as a 2.9-kb fragment (Figure 5A). Analysis of X-Gal-stained transient transgenic embryos at E14 revealed that the region surrounding ECR-VII targeted reporter activity to the MEE/periderm (Figure 3, and data not shown). In contrast, the 7.6-kb region containing both ECRs -V and -VI was able to drive expression not only in the MEE/periderm with high efficiency (7/14) (Figures 6B, 6C, 6V,6W), but also in the vasculature (including palatal vessels), nostrils and whisker follicles (Figures 6C–F). Within this region, ECR-V is conserved only in mammals but ECR-VI is conserved in both mammals and avians suggesting that ECR-V would be more likely to contain palate-specific control elements. Indeed, this was the case, since the sequences between −9.6 and −6.1 kb including ECR-V targeted reporter activity to the MEE/periderm (Figure 3 and Figures 6G–K, X, Y), while ECR-VI and surrounding sequences (from −13.7 to −9.7 kb) did not (Figure 3 and data not shown). To narrow down the regions within −9.7 and −6.1 kb that contained putative cis-regulatory modules, we next examined a 0.3-kb fragment that encompassed the highly conserved ECR-V (from −7.9 to −7.6 kb), and an adjacent conserved 0.8-kb region from −7.4 to −6.6 kb (Figure 6A). Each region drove reporter expression in the tips of palatal shelves but relatively weakly (Figures 3, 6L–U) and with far less specifically than the larger (−13.7 to −6.1 kb) fragment. These results suggest that the 3.5-kb region in Ift43 intron 2 contains two or more cis-regulatory elements independently able to direct the reporter activity in the MEE and adjacent periderm, but they are needed in combination to drive expression efficiently.

AN ADDITIONAL CIS-REGULATORY REGION IS LOCATED IN A 5.3-kb FRAGMENT IMMEDIATELY UPSTREAM OF Tgfb3 EXON 1

Since the overall conservation of the intergenic region between the Ift43 and Tgfb3 genes is relatively high, we examined whether this region could also contribute to MEE/periderm-specific expression (Figure 7A). First we cloned the 9.8-kb region from −6.1 to +3.7 kb (i.e., Ift43 intron 1, Ift43 exon 1, intervening sequences between the Ift43 and Tgfb3 genes and Tgfb3 exon 1) between concatamerized pairs of CHS4 insulators, and inserted a lacZ-PA cassette in frame into the Tgfb3 exon 1 (Figure 7B). This construct, driven by the endogenous Tgfb3 promoter, directed reporter activity specifically in the palatal midline region in transient transgenic embryos, although with a relatively low frequency (2/5) (Figures 3B, 7B–F). To further define the important region within this 9.8 kb fragment, we subcloned the 5.3-kb region from −6.1 to −0.8 kb into the 2xcHS4-hsp68-lacZ-2xcHS4 vector, as it lacks the endogenous Tgfb3 promoter (Figure 7G). Two of the three resulting transgenic embryos showed reporter activity
in the palatal midline (Figure 7H) though also in several other tissues (Figures 3, 7I–N), suggesting that the shorter sequence lacked elements necessary for highly regionally specific regulation. Introns 1 of Ift43 (from −6.1 to −3.7 kb) alone, or the intervening sequence between Ift43 and Tgfb3 (from −3.7 to −0.8 kb), did not direct β-galactosidase activity in the tips of palatal shelves (n = 5 in each case) (Figure 3B and data not shown). These data imply that a fragment from Ift43 intron 1 to Tgfb3 exon 1 contains a putative proximal MEE/periderm enhancer, which is dependent on DNA sequences separately located in smaller fragments.

**DISCUSSION**

Conditional Cre drivers are an invaluable tool for investigating the roles and timing of gene expression in processes involving several cell types such as palatogenesis. Key to this is knowledge of their recombination patterns and efficiency. Here we have shown that K14-Cre recombines efficiently in palatal medial edge epithelium (MEE) but it is not expressed in the overlying palatal periderm. This could explain the phenotypic differences between the germline Tgfb3 mutants (in which no Tgfb3 is expressed by MEE or periderm, periderm is inadequately shed and a complete cleft of the secondary palate occurs) and the epithelium-specific Tgfb3:K14-Cre mutants (in which genotypically normal periderm itself may be providing sufficient TGF-β3 signaling for some shedding, and thus a milder phenotype occurs). This proposed role for peridermal Tgfb3 expression, and known peridermal responsiveness to TGF-β3-triggered signaling (Wu et al., 2013) combined with an inability of K14-Cre to recombine in peridermal cells could also explain why periderm behaves normally in Tgfbr2:K14-Cre mutants (Iwata et al., 2013). In contrast with these TGF-β3 signaling, K14-Cre conditional knock-outs, epithelium-specific β-catenin mutants (Ctnn1b:K14-Cre) lose Tgfb3 expression in tips of palatal shelves but still develop total cleft of the secondary palate (He et al., 2011), raising the intriguing possibility that canonical Wnt signaling is specifically required in MEE for Tgfb3 expression to occur in both the MEE and adjacent periderm. To test these and related hypotheses other conditional Cre drivers are required: to recombine only in palatal periderm, and to recombine in both MEE and the overlying periderm. It is not clear whether all other Cre-drivers currently used to delete genes in the palatal epithelium recombine in periderm as well, and many have additional limitations: Pitx2-Cre recombines predominantly in the posterior palatal epithelium (Xiong et al., 2009); recombination in the Foxg1-Cre line is highly background-dependent.
FIGURE 6 | Cis-regulatory elements targeting reporter activity to the MEE and adjacent periderm are located in intron 2 of the upstream Ift43 gene. (A) Schematic representation of a 28-kb sub-region of the 61-kb genomic fragment (a, see Figure 4 and the main text) that includes Tgfb3 exon 1 (red box) and exons 1 and 2 of the Ift43 gene (blue boxes), aligned with (b) evolutionary sequence conservation among selected vertebrate species (UCSC genome browser), and (c) among placental mammals (UCSC genome browser), and (d) RNA-seq profile in mouse palate at E14.5 (FaceBase Enhancer Project; A Visel) used to identify the positions of non-coding evolutionary conserved regions (ECRs-V, -VI, and -VII: gray boxes). Colored bars below (d) correspond to DNA fragments (see also Figure 3) examined by transient transgenic reporter assay in constructs shown schematically above images of the stained embryos generated (B–Y). (C–F) Transgenic reporter embryos carrying a 7.6-kb DNA fragment from −13.7 to −6.1 kb (green bar below (c) and green rectangle in construct schematic) showing β-gal activity (blue staining) in tips of palatal shelves (C, black arrows), blood vessels (D, green arrows) and nostrils (F, red arrow). (H–K) Transgenic reporter embryos carrying a 3.6-kb DNA fragment from −9.7 to −6.1 kb (green-blue bar below (c) and green-blue rectangle in construct schematic) showing β-gal activity (blue staining) in tips of palatal shelves (H, black arrows), lens (J, blue arrow) and nostrils (K, red arrow). (M–P) Transgenic reporter embryos carrying a 0.3-kb DNA fragment (ECR-V) from −7.9 to −7.6 kb (light blue bar below (c) and light blue rectangle in construct schematic) showing β-gal activity (blue staining) in tips of palatal shelves (M, black arrows), apical ectoderm (N–P) and nostrils (P, red arrow). (R–U) Transgenic reporter embryos carrying a 0.8-kb DNA fragment from −7.4 to −6.6 kb (turquoise bar below (c) and rectangle in construct schematic) showing β-gal activity (blue staining) in tips of posterior palatal shelves (R, black arrows). (V–W) Frontal sections of the X-Gal-stained 7.6-kb fragment transgenic embryo shown in (C) at the level of the posterior palate (indicated by the red line in C). Staining at the tips of palatal shelves (black arrows in V) is in both MEE cells (red arrow in W) and periderm cells (black arrow in W). (X,Y) Frontal sections of the X-Gal-stained 3.6-kb fragment transgenic embryo shown in (H) at the level of the posterior palate (indicated by the red line in H). Staining at the tips of palatal shelves (black arrows in X) is in both MEE cells (red arrow in Y) and periderm cells (black arrow in Y). Scale bars in C,H,M,R, 500 μm; D,I,N,S,E,F,J,K,O,P,T,U, 1 mm; V,X, 100 μm; W,Y, 50 μm.
FIGURE 7 | A putative proximal enhancer directing palatal expression lies in a 5.3-kb region upstream of the Tgfb3 gene. (A) Schematic representation (a) of a 28-kb region upstream of Tgfb3 exon 1 (red box) including Ift43 exons 1 and 2 (blue boxes) aligned with (b) evolutionary conservation among selected vertebrate species (ucsc genome browser), (c) evolutionary conservation among the placental mammals (ucsc genome browser), (d) RNA-seq profile in mouse palate at E14.5. Colored bars below (d) correspond to DNA fragments examined using transient transgenic reporter assays (B–N). (B,G) Schematic presentations of reporter constructs used to generate transgenic embryos [colored rectangles correspond to colored bars shown above (a)]. (C–F) Transgenic reporter embryo carrying the 9.8-kb DNA fragment from −6.1 to +3.7 kb (magenta bar above (a) and rectangle in B) showing β-gal activity (blue staining) in tips of palatal shelves (C, black arrows) and in the nasal septum (asterisk in C). No staining was seen in the torso (D) or head (E,F). (H–K) Transgenic reporter embryo carrying the 5.3-kb DNA fragment from −6.1 to −0.8 kb [dark purple bar above (a) and rectangle in G] showing β-gal activity (blue staining) in tips of palatal shelves (H, black arrows), in nasal septum (asterisk in H), in the ectoderm (red asterisks in H), and in skeletal structures (blue arrows) and olfactory bulbs (J, red arrows). (L–N) Frontal sections of the X-Gal-stained 5.3-kb transgenic embryo shown in (H) at the level of the nasal septum (indicated by the red line in H). Staining can be seen at the tips of palatal shelves (black arrows in L,M) and in periderm cells of the nasal septum (black arrow in N). Scale bars in (C,H), 500 μm; (D,I,E,F,J,K), 1 mm; (L), 200 μm; (M,N), 50 μm.

(Hebert and McConnell, 2000); and, as Tgfb3 itself is expressed and required in several other tissues besides MEE and adjacent periderm during early embryogenesis, the Tgfb3-Cre knock-in line is of very limited use in studies of palatal epithelial fusion (Yang et al., 2008). As expression of Tgfb3 in MEE and in periderm is so crucial to normal palatogenesis, occurs in precisely the regions where we would like to regulate the expression of other genes genetically, and its regulation poorly understood, we set out to identify the enhancer sequences responsible for this highly specific expression of Tgfb3. Using BAC deletion analysis we were able to identify a 61-kb region around the Tgfb3 gene that could drive lacZ reporter expression specifically in the MEE and adjacent periderm. Expression of this reporter was much more specific to the MEE/periderm than that of the endogenous Tgfb3 gene, and as this 61-kb region did not drive detectable expression before E12.5 it is a good candidate region for the development of novel palatal epithelium/periderm-specific Cre-driver lines.

In order to use enhancer sequence information to learn more about molecular regulation of Tgfb3 expression we needed to identify the important sequences more precisely. Palatogenesis is an evolutionarily conserved developmental process in amniote animals (Bush and Jiang, 2012). Mammals and reptiles have a fused secondary palate, and although avians develop a beak...
and have a naturally cleft palate (Ferguson, 1988) fusion can be induced by exposing the appropriate stage avian palatal shelves (which do not express endogenous cTgfβ3) to human recombinant TGF-β3 (Sun et al., 1998). Although it was therefore likely that enhancers directing palatal expression would be amongst non-coding highly conserved sequences amongst mammals, these are very numerous, and those that lay within the Tgfβ3 gene (ECRI-IV) turned out not to be palatal enhancers. The FaceBase project (www.facebase.org -A. Visel) to identify craniofacial transcriptional enhancers using ChIP-Seq (IP using anti-p300) recently released a dataset obtained on E14 whole palates but this was not helpful for our specific project; within the 61-kb region only a region around Tgfβ3 exon 1 was flagged as being a putative enhancer; we could demonstrate only a putative vascular enhancer in the area of ECR-VII (Figures 3, 4 and data not shown) where the anti-p300 binding was above the background level in ECR-VII (Figure 4).

By directing our analysis outside the Tgfβ3 gene within the 61 kb fragment, we were able to identify a distal 3.5-kb region in Ift43 intron 2 and a proximal 5.3-kb region encompassing Ift43 intron 1 and most of the intergenic sequence between the Ift43 and Tgfβ3 genes able to target the reporter activity to the MEE and adjacent periderm. However, these smaller regions directed less specific and weaker reporter activity than the 61 kb fragment. While we were able to break the distal 3.5-kb region down further into two smaller modules, which again showed further reduced activity, our attempts to narrow down the 5.3-kb proximal region into even shorter sequences were not successful, suggesting that palate-specific reporter activity seen in the larger region was dependent on two or more regulatory elements separately located in the smaller fragments. Although the same approach has yielded relatively short enhancers that drive very precise and strong expression in other cases (Dodou et al., 2004; Chandler et al., 2007), it is established that not all physically concise and robust expression is regulated in such a simple manner (Evans et al., 2012). Genetic regulatory network studies in Drosophila first introduced the concept of “shadow” enhancers (Lagha et al., 2012). Perry et al. reported that, in addition to the proximal primary enhancer located just upstream of the promoter, the snail gene is regulated by a distal enhancer located within the neighboring locus (Perry et al., 2010), which they suggested be defined as a “shadow” enhancer. Subsequent studies have suggested that secondary enhancers are needed to obtain sufficient phenotypic robustness to drive tightly controlled expression of important developmental genes (Frankel et al., 2010). Our findings of putative proximal (primary) enhancer(s) and two (or more) distal enhancers in the neighboring upstream gene that work precisely but only weakly in isolation are reminiscent of this mechanism. A “lack of simplicity” may also extend to the organization of enhancers for other tissues and repressive elements controlling Tgfβ3 expression as we noticed that, unlike the 61-kb region which directed reporter activity specifically in the MEE/periderm, many of the smaller domains around the ECR-V also targeted the reporter activity to the vasculature including the palatal vessels. Similar vascular patterns were seen in embryos carrying either ECR-VI or ECR-VII (which were unable to direct expression in the MEE/periderm) suggesting that all three ECRs located in intron 2 of Ift43 possess putative redundant vascular enhancer activities.

Very little is known about the molecular mechanisms regulating Tgfβ3 expression in the epithelial tips of pre-fusion palatal shelves. Venza et al. recently reported that in Foxe1 mutant embryos Tgfβ3 expression is dramatically reduced in the palatal epithelium, and that Tgfβ3 is a direct target of FoxE1 via FoxE1 binding sites in the Tgfβ3 promoter region (Venza et al., 2011). As outlined above, He et al. reported that epithelium-specific mouse mutants lacking the gene encoding β-catenin also show a dramatic reduction in Tgfβ3 expression in the palatal epithelium suggesting that canonical Wnt signaling is involved in Tgfβ3 regulation (He et al., 2011). Whether these identified transcriptional regulators function purely by contributing to the core promoter activity or by also regulating Tgfβ3 expression via distal enhancers is not yet known. Nevertheless, even the smallest cis-regulatory region identified in this study (i.e., the 300-bp ECR-V located in the Ift43 intron 2) contained three evolutionarily conserved TCF/LEF consensus binding sites and two FoxE1 binding sites (data not shown) implying that these factors may have the capacity to regulate Tgfβ3 in palatal shelf tissues by binding directly to the putative enhancer elements. Thus our results are consistent with existing molecular regulation data, and suggest a model in which the MEE/periderm-specific Tgfβ3 expression is achieved via a complex regulatory landscape composed of a putative proximal (primary) enhancer(s) and two (or more) distal shadow enhancers i.e., some that lie in the neighboring upstream gene.

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