The BMP Ligand Gdf6 Prevents Differentiation of Coronal Suture Mesenchyme in Early Cranial Development

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Abstract

Growth Differentiation Factor-6 (Gdf6) is a member of the Bone Morphogenetic Protein (BMP) family of secreted signaling molecules. Previous studies have shown that Gdf6 plays a role in formation of a diverse subset of skeletal joints. In mice, loss of Gdf6 results in fusion of the coronal suture, the intramembranous joint that separates the frontal and parietal bones. Although the role of GDFs in the development of cartilaginous limb joints has been studied, limb joints are developmentally quite distinct from cranial sutures and how Gdf6 controls suture formation has remained unclear. In this study we show that coronal suture fusion in the Gdf6+/- mouse is due to accelerated differentiation of suture mesenchyme, prior to the onset of calvarial ossification. Gdf6 is expressed in the mouse frontal bone primordia from embryonic day (E) 10.5 through 12.5. In the Gdf6+/- embryo, the coronal suture fuses prematurely and concurrently with the initiation of osteogenesis in the cranial bones. Alkaline phosphatase (ALP) activity and Runx2 expression assays both showed that the suture width is reduced in Gdf6+/- embryos and is completely absent in Gdf6-/- embryos by E12.5. ALP activity is also increased in the suture mesenchyme of Gdf6+/+ embryos compared to wild-type. This suggests Gdf6 delays differentiation of the mesenchyme occupying the suture, prior to the onset of ossification. Therefore, although BMPs are known to promote bone formation, Gdf6 plays an inhibitory role to prevent the osteogenic differentiation of the coronal suture mesenchyme.

Introduction

The mammalian cranial vault is composed of five main flat bones separated by joints known as the cranial sutures. These sutures are composed of fibrous connective tissue and act as the main sites for cranial growth during development. As the cranial vault expands, bone is deposited at the growing edges of the bone (the bone fronts), while the suture mesenchyme remains undifferentiated. Sutures provide flexible joints for passage through the birth canal, as shock absorbers, prevent separation of the cranial bones, and accommodate room for the rapidly growing brain [1]. With the exception of the metopic suture, human sutures normally do not fuse until the third or fourth decade of life [2], when the undifferentiated mesenchyme of the suture space becomes obliterated by bone.

Craniosynostosis is defined as the premature fusion of one or more of the cranial sutures and occurs in approximately 1 in 2,500 live births [3]. When a suture fuses prematurely, cranial growth ceases perpendicular to the fused suture, producing a dysmorphic skull shape. In turn, when the calvarial vault cannot expand sufficiently to accommodate the rapidly growing brain, increased intracranial pressure can occur [4]. Coronal craniosynostosis can result from several potential mechanisms. For example, a failure to form the developmental boundary between the neural crest-derived frontal bone and the paraxial mesoderm-derived parietal bone can result in impaired suture formation. This failed mechanism is evident as a mixing of the frontal and parietal cell populations at sites of suture fusion in utero, as seen in the Twist1 mutant mouse [5]. It is thought that Twist1 functions with Msx2 to control the localization of ephrin-A2 and ephrin-A4, which are known to play roles in boundary formation at the frontal/parietal junction by restricting cell migration [5]. Several additional mechanisms could also lead to fusion of a cranial suture. These include changes in proliferation, apoptosis, or the rate of differentiation in the suture mesenchyme or at the leading edges of the ossifying bone. For example, gain of function mutations in Fibroblast growth factor receptors (FGFRs) have been associated with craniosynostosis in humans. Studies in mice have shown that Fgfr2 is expressed in proliferating osteoprogenitor cells surrounding the ossifying bones while Fgfr1 is expressed more centrally in osteoid of the developing frontal and parietal bones. As differentiation progresses, Fgfr2 is downregulated and Fgfr1 is upregulated, suggesting that signaling through FGFR2 mainly plays a role in proliferation, while FGFR1 signaling regulates osteogenic differentiation. The contribution of FGFR3 is less clear though its expression overlaps with FGFR1 and -2 [6]. However, the P250R gain-of-function mutation in FGFR3 has been associated with coronal craniosynostosis, either isolated or as part of syndromes such as Muenke syndrome [7]. While defects in boundary formation between lineage compartments (e.g. neural crest and paraxial mesoderm) can explain some of the etiology of coronal craniosynostosis, it remains less clear how proposed
Changes in differentiation or maintenance can affect certain sutures while sparing others.

Growth Differentiation Factors (GDFs) 5, 6, and 7 are members of the Bone Morphogenetic Protein (BMP) family of secreted signaling molecules. The GDF subgroup (GDF5/6/7) is highly conserved in vertebrates and has been shown to play a critical role in limb joint formation and chondrogenesis [8]. Gdf6 homozygous knockout mice display multiple joint defects, including fusions of tarsal and carpal bones, morphological abnormalities in the malleus, incus, and stapes bones of the middle ear, and hypoplasia of the thyroid cartilage. In addition to these defects, Gdf6 knockout mice lack the coronal suture [9]. However, the detailed expression pattern of Gdf6 in the developing skull and its relationship to the onset of cranial suture fusion in this mutant has not been shown. Another GDF family member, Gdf5, is mutated in the brachycephaly mouse and normally stimulates cartilage development, growth, and maturation [8]. Therefore, Gdf5 can be viewed as promoting aspects of endochondral bone growth. Gdf5 and Gdf6 share approximately 80% identity in the mature signaling region [10] and therefore it is likely the Gdf5 and Gdf6 operate by similar ligand-receptor interactions. Like Gdf5, Gdf6 (a.k.a. CDMP2) can promote chondrogenic differentiation in vitro [11]. This makes the craniosynostosis phenotype in the Gdf6+/− mouse particularly interesting because unlike the long bones, the cranial bones form through intramembranous ossification without a cartilage intermediate. Therefore, the mechanism of Gdf6 function in the coronal suture may be drastically different than its function in joints of the axial skeleton.

The aim of this study was to gain a more thorough developmental understanding of craniosynostosis in the Gdf6+/− mouse and the underlying cause of suture fusion. We found that the coronal suture is obliterated in Gdf6+/− embryos before the first evidence of cranial bone ossification is detectable at E14.5, with changes in early osteogenic markers detected prior to the onset of ossification. Our data suggests that Gdf6 may self-regulate its expression in the developing frontal bone primordium. Additionally, fusion in the Gdf6+/− is not due to a failure to form the boundary properly between the frontal and parietal bones, or changes in cell survival or proliferation, but is likely due to a failure of the suture mesenchyme to remain in an undifferentiated state.

Results

Gdf6+/− Coronal Suture Fuses Early in Development

The entire coronal suture was absent in Gdf6+/− fetal mice (Fig. 1C, F), with complete penetrance (not shown). This defect was not observed in wild-type (Fig. 1A, D) and Gdf6 heterozygote littermates (Fig. 1B, E) through embryonic development and weaning. To determine the time point during development at which the suture first became fused, Gdf6+/− embryos were collected at various stages and stained with alizarin red. At E14.0, ossification centers in either the frontal or parietal bone were not yet visible by alizarin red staining. By E14.5, frontal and parietal bones were first visible as two separate ossification centers (Fig. 1G, H, K). The nascent coronal suture was apparent in the wild-type embryo as the gap between the two bones. Yet in Gdf6+/− embryos, a single continuous ossification center was present (Fig. 1I, L). Analysis of multiple Gdf6+/− embryos at E13.5–14.5 failed to identify visibly separate sites of alizarin staining for frontal and parietal rudiments in any specimens (not shown). At E15.5, the frontal and parietal bones were fused into one continuous bone in the Gdf6+/− embryo (Fig. 1O), while the bones remained separated by the coronal suture in the wild-type and heterozygote (Fig. 1M, N) embryos. The coronal suture continued to fuse along its entire length as ossification progresses outward in the Gdf6+/− embryo, whereas the sagittal, lambdoid, and squamosal sutures (Fig. 1A–F) remained unaffected in Gdf6+/− mice throughout prenatal development. At the macroscopic level, the ossification centers and suture in Gdf6+/− appear to develop identically to wild-type embryos with regards to the onset of ossification and size of the bones (not shown). This data suggests that Gdf6 plays a role in coronal suture formation at or prior to the onset of ossification.

Gdf6+/− Has Normal Suture Boundary Formation

Cranial craniosynostosis can result from a failure to form a proper boundary between cells of the neural crest-derived frontal bone and the paraxial mesoderm-derived parietal bone. The formation of this boundary involves the cooperation of Twist1 and Mix2 to control the expression domains of ephrin-A2, ephrin-A4, and EphA4 [5]. Ephrin signaling has been shown to inhibit cell mixing and provide guidance cues for migrating cell populations [12]. Failed boundary formation is evident as a mixing of the two cell lineages within the suture mesenchyme, as in the Twist1 mutant mouse [5].

To determine whether a similar cell mixing was the cause of the craniosynostosis in the Gdf6+/− embryos, we visualized the suture boundary using the Wnt1-Cre and R26R transgenic lines, which together stably label derivatives of the neural crest [13], including the frontal bone. At E16.5, coronal suture fusion was evident in whole-mount stained Gdf6+/− embryos (Fig. 2A, B). Transverse sections showed the presence of ossified bone across the boundary between the frontal and parietal bones in Gdf6+/− (Fig. 2D, arrow), while the suture remained open and undifferentiated in wild-type (Fig. 2C, arrow). Also of note was a general thinning of the bone in this region of the Gdf6+/− (Fig. 2D) along with the loss of the characteristic suture mesenchyme blastema, seen in wild-type embryos (Fig. 2C, arrow). In the regions of the frontal bone more distal to the suture, there was no thinning of the Gdf6+/− bone compared to wild-type (not shown).

In the Gdf6+/− embryo, ossified tissue disrupted the boundary between the frontal and parietal domains (as seen in Fig. 2D) making it difficult to determine if cells have crossed the normal boundary. Therefore embryos were examined at E14.5, when portions of the Gdf6+/− calvaria were not yet ossified through the suture. Coronal sections through the region of the presumptive coronal suture reveal that although the Gdf6+/− lacked an identifiable suture, the cellular boundary between the frontal and parietal bones remained distinct just like the wild-type suture (Fig. 2E, F). We could find no evidence of cell mixing between these tissue populations and there were no other obvious differences between wild-type and Gdf6+/− embryos with regards to the suture boundary (not shown). Furthermore, we observed a surprisingly uniform and continuous surface between the frontal and parietal bones of Gdf6+/−, where the suture should reside.

Gdf6+/− and Gdf6+/− Present with Pre-Ossification Changes in the Suture

Transverse sections through the E12.5 wild-type suture were stained for alkaline phosphatase (ALP) activity, an early osteoblast marker, to highlight the frontal and parietal bone primordia with the presumptive coronal suture in between (Fig. 3A). ALP was weaker in the band of cells between the primordia, but was faintly visible across the suture region (Fig. 3A). In Gdf6+/− embryos, ALP activity extended through the presumptive suture region which was shorter as defined by the distance between the flanking bone primordia (Fig. 3B), revealing less undifferentiated suture.
mesenchyme relative to the wild-type suture. Although Gdf6+/− mice develop normal sutures, ALP was also increased in the presumptive suture relative to wild-type. This points to a dosage effect, where loss of one copy of Gdf6 does increase ALP activity in the suture, but this slightly increased differentiation does not reach the threshold required for fusion of the suture. In addition, the suture gap width was reduced in the Gdf6+/− compared to wild-type (Fig. 3B). In E12.5 Gdf6−/− embryos, the frontal primordium was clearly visible, but there was no clear region of concentrated ALP in the parietal primordium; rather, there was continuous, moderate ALP staining across the region of the presumptive suture and extending into the region where the parietal primordia has concentrated ALP activity in wild-type and Gdf6+/− embryos (Fig. 3C).

Differentiation of the suture mesenchyme was also examined by expression of Runx2, another marker for early bone differentiation. Like ALP, Runx2 marked the presumptive frontal and parietal bones, with a gap of undifferentiated mesenchyme in between (Fig. 3D). Again, the distance between the frontal and parietal rudiments was reduced in the Gdf6+/− (Fig. 3E). In the Gdf6−/−, Runx2 was expressed continuously through the suture region at E12.5 (Fig. 3F). These data suggest the presumptive suture region...
of Gdf6−/− embryos is prematurely differentiating by E12.5, as characterized by abnormally high ALP and Runx2 expression. Interestingly, ALP activity is apparently delayed in the parietal rudiment of Gdf6−/− embryos although Runx2 expression is not.

Increased ALP activity was confirmed at both caudal (closer to the eye) and rostral (newly formed) levels of the presumptive coronal suture at E13.5 and E14.5 (caudal, Fig. 3J–L, P–R and rostral, Fig. 3G–I, M–O). This suggests an increase in osteogenic differentiation of the cells in the developing suture of the Gdf6+/− and Gdf6−/− embryos, and also a dosage effect where Gdf6+/− has intermediately increased differentiation between wild-type and Gdf6−/− embryos.

A decrease in both the intensity (Fig. 3C,I) and span (not shown) of ALP activity in the parietal bone rudiment of Gdf6−/− embryos was noted, suggesting a decrease in the rate of differentiation in this structure. This was also observed in the alizarin red staining of E14.5 embryos, with a reduction of the size of the Gdf6−/− parietal bone ossification center compared to wild-type and Gdf6+/− (not shown). This could be a potential secondary effect of loss of the suture, or a direct effect of the loss of Gdf6.

Loss of Gdf6 does not Significantly Affect Proliferation in the Nascent Suture

In principle, suture fusion could result from changes in the number of cells in the suture or bone fronts proliferating or undergoing apoptosis, thereby increasing or decreasing the number of cells in the pre-osteogenic pool. Between E14.0 and E14.5, the frontal and parietal rudiments in the Gdf6−/− are clearly fused as shown by alizarin red staining into a single ossified element (Fig. 1I, L). Therefore the sutures of wild-type, Gdf6+/−, and Gdf6−/− littermates were examined for changes in proliferation or apoptosis at E12.5, concurrent with the earliest detectable changes in ALP staining (Fig. 3) but before the formation of osteoid. Adjacent sections were stained for ALP in order to help localize the suture (Fig. 4A–C). Immunohistochem-
Gdf6 Prevents Coronal Suture Differentiation

Gdf6 is Expressed in the Frontal Bone Primordia

The neural crest/paraxial mesoderm tissue boundary that determines the future site of the coronal suture is formed by E10.5 [13]. In order to pinpoint the pattern of Gdf6 expression during cranial development and specifically in relation to coronal suture formation, we performed in situ hybridization on embryos at E9.5–E14.5. Gdf6 mRNA was first detected in the developing cranial region in a triangular-shaped area just anterior to the eye at E10.5 (Fig. 5A–C, arrow). This corresponded closely with the neural crest-derived frontal bone rudiment, as labeled in Wnt1-Cre; R26R embryos (Fig. 5D, arrow). Gdf6 was expressed in the frontal bone rudiment at E11.5 (Fig. 5H–J) as the rudiment begins to grow and expand. Transverse sections through the E10.5 frontal rudiment (depicted in Fig. 5E, dotted line) reveal that the site of expression anterior to the eye was localized to several layers of mesenchyme underlying the surface ectoderm (Fig. 5F,G). At E12.5, Gdf6 continued to be exclusively expressed in the frontal bone rudiment (Fig. 5K–M, arrow), with no evidence of expression in the suture mesenchyme or parietal bone rudiment. By E14.5, when fusion of the ossification centers was first visible by alizarin red staining, Gdf6 is no longer expressed in the suture region (Fig. S2).

Although no functional Gdf6 protein can be made from the Gdf6 knockout allele, the mutant Gdf6 transcript can be detected by our in situ RNA probe, which targets the 3’ UTR of Gdf6. In situ hybridization revealed that Gdf6 transcripts were still present in Gdf6−/− embryos (Fig. 5C,J,M). Interestingly, the Gdf6 transcript was more highly expressed in Gdf6+/− embryos than in either wild-type or Gdf6+/-, with staining in Gdf6+/- at intermediate levels (Fig. 5A–C, H–J). Increased staining was also observed for Gdf6 in the branchial arches of Gdf6−/− embryos (Fig. 5, A–C, H–J asterix). These observations suggest that Gdf6 expression may be self-regulated in these structures by a negative feedback loop. In cross-section, Gdf6 expression was also observed in the orbital bone rudiment (Fig. 5L) of wild-type, Gdf6+/−, and Gdf6−/− embryos (not shown). Gdf6 is also normally expressed in the dorsal retina where it functions in promoting eye development [15,16]. However, Gdf6 transcript was observed in the dorsal retina wild-type and Gdf6+/− embryos (Fig. 5K,L), but was lost in Gdf6−/− embryos (Fig. 5M), suggesting that in the retina Gdf6 autoregulates through a positive feedback loop, in contrast to the negative autoregulation in frontal bone and branchial arches.

Discussion

Here we present data indicating that Gdf6 is genetically required for osteogenic differentiation in the developing coronal suture during its formation. Gdf6 is absolutely required for formation of the suture, as in Gdf6−/− embryos there is an initial failure to establish a region of delayed differentiation between the frontal and parietal condensations. We observed that Gdf6 mRNA is strongly downregulated in wild-type calvaria by E14.5. It remains unknown if Gdf6 plays roles at later stages to maintain patency of

Figure 4. Analysis of cell proliferation in the E12.5 coronal suture. Adjacent sections stained for ALP activity, highlighting the location of the frontal and parietal bones (A–C dotted lines), and immunohistochemistry for phospho-histone H3 (A’–C’). Examples of positive cells are marked with arrows. (D) Quantification of the mean number of proliferating cells counted in the combined frontal/suture/parietal region. (E) Quantification of the mean number of proliferating cells counted in each region separately (the frontal bone, parietal bone, and suture mesenchyme) (x-axis). Since the suture mesenchyme cannot be distinguished from the parietal in Gdf6−/− embryos at this time, only data for the frontal rudiment is shown for this genotype. N = 3 embryos for each genotype with 5 sections per embryo quantified. Differences the number of proliferating cells per suture region were analyzed using a t-test.

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the established suture; such a notion would be consistent with a report that Gdf6 expression is detectable in calvarial sutures by E16.5 [9]. The question of whether Gdf6 plays a role in suture maintenance will require examination of Gdf6 protein stability and localization, and/or conditional deletion of Gdf6 at later stages.

Gdf6 Represses Osteogenic Differentiation but not Boundary Formation in the Coronal Suture

We found no evidence for disruption in the frontal/parietal cell boundary in Gdf6−/− mice. This is in contrast to the mechanism of suture fusion in the Twist+/−, and Epha4−/− mice where, before E14.5, osteogenic cells from the frontal bone abnormally cross into the suture mesenchyme [5]. In Twist+/− mice, ALP activity in the frontal/parietal rudiments is normal in appearance up to at least day E13.5 [5]. In contrast, ALP activity abnormalities are detectable by E12.5 in Gdf6−/− embryos, before ephrin ligands are expressed in the frontal/parietal region [5]. Therefore Gdf6 is required for a mechanism of suture formation that is distinct from that controlled by the Twist/Ephrin pathway. Twist also regulates osteogenic condensation via interaction with Msx2 [17]. Our data does not exclude Gdf6/Twist interactions during osteogenic differentiation, although a combined reduction of Twist and Msx2 levels was shown to primarily affect differentiation of the frontal, but not the parietal bone, suggesting this is possible [17].

Effects of Gdf6 Signaling in the Developing Calvarium

Somewhat surprisingly, Gdf6 mRNA was not detected in the suture mesenchyme itself but in the frontal bone primordia. This is in contrast to the sites of wrist and ankle joint fusion in Gdf6−/− embryos, where Gdf6 is clearly expressed in the developing joint interzones [9]. However, several studies suggest that the action of GDFs in limb joint development is not explained by direct autocrine suppression of chondrogenesis; for example, both the direct application of GDF5 protein to developing limb cartilage and transgenic Gdf5 overexpression are pro-chondrogenic [8,18], although in limb joints the pro-chondrogenic effects of GDFs are probably inhibited by Noggin [19].

Gdf5/6/7 form a closely related subfamily of BMPs, sharing 80% identity in their mature C-terminal signaling domains [10]. The strong similarity of Gdf5 and Gdf6 suggest they share similar signaling properties. Genetic evidence from analysis of Gdf5/Gdf6 compound mutant mice supports the idea that they have similar, partly redundant roles in skeletal development that are determined largely by site of expression rather than distinct signaling mechanisms [20]. A unifying theme of both limb joints and the frontal bone is that both are important paracrine signaling centers for adjacent targets (that is, cartilage in the limb and the suture mesenchyme in the

Figure 5. Expression of Gdf6. In situ hybridization for Gdf6 at E10.5 (A–C, F,G), E11.5 (H–J), and E12.5 (K–M). Gdf6 was expressed in the frontal bone primordia (arrow), which is labeled as neural crest-derived in the Wnt1-Cre; R26R transgenic embryos (D). Gdf6 transcript was expressed more strongly in the Gdf6−/− embryo (C, J) than either the wild-type (A, H) or Gdf6+/− embryo (B, I), in both the frontal bone rudiment (arrow) and branchial arches (asterix). In transverse cross section through the eye and frontal bone primordia at E10.5 (dotted line, E), Gdf6 was expressed in several layers of mesenchyme underlying the surface ectoderm (F, G). At E12.5, Gdf6 continues to be expressed in the frontal bone rudiment, in addition to the dorsal retina (K–L) and orbital bone rudiment (L). Expression of Gdf6 in the dorsal retina seen in the wild-type and Gdf6+/− (KL) was absent in the Gdf6−/− (M). dr, dorsal retina; e, eye; fr, frontal rudiment; or, orbital bone rudiment.

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Gdf6 Prevents Coronal Suture Differentiation
Gdf6 expression in the frontal bone primordium enables it to serve as a paracrine signaling center to affect cellular processes in the suture mesenchyme and the parietal rudiment. It is not yet clear if this occurs via direct signaling or through indirect effects transmitted by downstream effectors.

Interestingly, the onset of ALP activity is delayed in the parietal, but not frontal, primordia in Gdf6−/− embryos. Since Gdf6 is expressed in the frontal but not parietal primordia, this suggests Gdf6 signaling from the frontal primordium also acts in a paracrine manner to influence maturation of the parietal. In this view, Gdf6 actually stimulates osteogenic maturation of the parietal rudiment, although it is not clear if this affect is direct or indirect (for example, it could be a secondary effect mediated by Gdf6 regulating a separate factor in the suture mesenchyme). Therefore, data regarding Gdf6’s possible roles in skeletal differentiation are important for interpretation of our results. Several reports indicate that like Gdf5, Gdf6 can stimulate chondrogenic differentiation in cell culture models [11]. Whether Gdf6 is pro- or anti-osteogenic is less clear. Some studies indicate that in vitro, Gdf6 can have pro-osteogenic effects on osteoblastic cells in similar manner to Gdf5, albeit its ability to induce osteoblast markers is much less than that of the “canonical” osteogenic BMPs such as BMP2 or BMP7 [21]. However, Gdf6 can inhibit ALP expression and mineralization in bone marrow-derived mesenchymal stem cells [22]. In vitro experiments must be interpreted with caution, due to potential differences in expression of BMP receptors and/or inhibitors across cell lines. In general, in vitro studies indicate that Gdf6 seems consistently capable of promoting differentiation of chondrogenic cells but relatively poor, or inhibitory, at promoting osteogenesis.

However, injection of GDF5 into perinatal mouse calvaria in vivo led to increased bone formation [23]. While this suggest GDF5 can have stimulatory effects on calvarial osteogenic differentiation, the different timing and context of Gdf5 application may not lead to the same effects as Gdf6 in the prenatal calvaria and Gdf6 may have distinct signaling effects compared to Gdf5, despite similar receptor usage [24]. Other studies indicate that the effects of Gdf6 on ALP induction are context dependent [25]. This effect might be mediated by receptor subunit combinations, interactions with inhibitors such as Noggin, or even heterodimerization with other BMP family members. Noggin is expressed in layers surrounding the developing frontal and parietal bones and can bind Gdf6 and inhibit its signaling ability [26]. Noggin can repress BMP signaling in the coronal suture [27], so it likely inhibits Gdf6-mediated signaling. However, Noggin is not required for suture formation, as Noggin−/− embryos do form coronal sutures (not shown).

Therefore, Gdf6 is probably dispensable for the function of Gdf6 in suppressing suture differentiation. However, our data would not exclude the possibility that the reduction of ALP activity in the Gdf6−/− E12.5 parietal bone could be caused by increased Noggin activity, secondary to a reduction of Gdf6 that normally suppresses Noggin. Xenopus GDF6 can heterodimerize with other BMPs, such as BMP2, in vitro [26,28] and other BMP heterodimers can have distinct and potent effects as compared to homodimers [29]. Therefore it is possible that Gdf6/BMP heterodimers have unique signaling properties.

Alternatively, it is possible that the relative temporal development of the frontal and parietal rudiments is critical, and that the relative delay of the parietal ossification sequence leads indirectly to failure of suture establishment. However, the onset of Runx2 transcription is not delayed in Gdf6−/− embryos, suggesting Gdf6 is not required for temporal control of Runx2 mRNA in calvarial rudiments. We postulate that the suture mesenchyme and the parietal rudiment may be differentially sensitive to Gdf6 signaling.

Gdf6 Autoregulation and Interaction with other BMPs

We observed that Gdf6 transcription is increased in frontal bone, but reduced in eyes, of Gdf6−/− embryos. This suggests differential, tissue-specific autoregulation of Gdf6. Interestingly, Gdf5 represses its own transcription in limb joints [8]. We propose that Gdf6 autoregulates itself in the frontal bone and that Gdf6 BMP signaling levels are fine-tuned during normal coronal suture development to coordinate proper differentiation and morphogenesis. Interestingly, Bmp4 and Gdf6 are coexpressed in both the dorsal retina and the frontal bone primordia [16]. Mutations in Bmp4 and Gdf6 independently disrupt eye development [15,30,31]. Although Gdf6 is expressed in the frontal bone rudiment, there is no evidence for a frontal bone defect in Gdf6−/−. This is likely due to compensation by Bmp4, Bmp4+/−; Gdf6−/− mice at E18.5 do in fact have a frontal bone defect, with the persistence of large fontanelles that is not observed in either single heterozygotes (data not shown; manuscript in preparation). We speculate that these two BMP ligands cooperate to regulate suture and/or calvarial development.

Conclusions

In summary, we found that Gdf6 is required to control an early stage of repressed osteogenic differentiation in the coronal suture. Not only does this suggest potential new mechanisms for this BMP family member in regulating bone growth, it nomimates Gdf6 as a candidate gene harboring mutations in individuals with coronal craniosynostosis. Gdf6 mutations in humans have been associated with eye and postcranial skeletal abnormalities although these effects are characterized by incomplete penetrance and phenotypic heterogeneity [15,32]. Interestingly, genomic lesions close to the GDF6 genomic region are associated with Nablus Mask-Like Facial Syndrome [33,34,35] which is a complex multigene deletion syndrome characterized by loss of a critical region just proximal to GDF6. In one of only two known patients where the genomic deletion included Gdf6, coronal craniosynostosis was observed [33,34]. We propose that some individuals having coronal craniosynostosis with unknown etiology may harbor mutations in Gdf6.

Materials and Methods

Mice, Crosses and Genotyping

The Gdf6−/− mouse [9] was a gift from Dr. David Kingsley and was backcrossed onto a C57BL/6 background for more than 10 generations. Since the loss of Gdf6 is perinatal lethal on this background (not shown), all time points were collected prenatally. For fate mapping experiments, Gdf6+/− mice were crossed to R26R+/− [36] to produce Gdf6+/−; R26R+/− (double heterozygotes), which were then crossed to Wnt1Cre+/− [37]; Gdf6+/− mice. Embryonic age was determined through detection of the vaginal plug, with noon of that day observed as E0.5. DNA samples for genotyping were isolated using tail snips from postnatal mice and yolk sacs from embryos, and processed as previously described [38]. The Gdf6 [9], R26R [36], and Wnt1-Cre [39] lines were genotyped by PCR analysis as previously described. The use of animals in this study was approved by the Vanderbilt University Institutional Animal Care and Use Committee as part of animal use protocol M/09/293, approved on 1/25/10 and 1/25/11.
Whole Mount Skeletal Preps

Mid-gestation mice from E14.5 to E18.5 were collected, organs removed and skinned. Each specimen was placed in a separate 50 ml conical tube and then soaked in 95% ethanol for 1 day. Specimens were agitated constantly on a shaker. Preps were then stained in alcian blue solution [20% glacial acetic acid, 0.015% alcian blue in 95% ethanol] for 14 days, destained in 95% ethanol for 1 day, then transferred into 1% KOH until cleared. Once cleared, the skeletal preps were placed in alcizarin red staining solution [0.00125% alcizarin red in 1% KOH] for 1 day. Skeletal preps were then transferred into graded changes of increasing glycerol for storage (15%, 30%, 50%, 70%, 90% glycerol made in 1 x PBS, 100% glycerol).

Whole Mount and Slide in situ Hybridization

The Gdf6 RNA probe was generated by cloning a PCR fragment using the primers 5'- AAGCATTGGAAGGAGGAT-GAAAGG- 3' and 5'- CGACCTCAGTAACCT-TAGTGTGGTCA -3', targeting the Gdf6 3' untranslated region, into the pGEM-Teasy vector (Promega). The Rans2 RNA probe was made using the primers and protocol described previously [40]. Hybridization was performed over night at 65°C, with embryos incubated in 200 ng/ml (whole-mount) or 30 ng of probe per slide (sections). For sectioning, embryos were equilibrated in 50% sucrose and then embedded in Tissue-Tek O.C.T. per slide (sections). For sectioning, embryos were incubated in 200 ng/ml (whole-mount) or 30 ng of probe per slide (sections). For sectioning, embryos were equilibrated in 50% sucrose and then embedded in Tissue-Tek O.C.T. compound (Sakura Finetek). Frozen sections were collected at 18 um. For each time-point, embryos were from the same litter and stained for an equal length of time. The Gdf6 RNA probe was generated by cloning a PCR fragment using the primers 5'- AAGCATTGGAAGGAGGAT-GAAAGG- 3' and 5'- CGACCTCAGTAACCT-TAGTGTGGTCA -3', targeting the Gdf6 3' untranslated region, into the pGEM-Teasy vector (Promega). The Rans2 RNA probe was made using the primers and protocol described previously [40]. Hybridization was performed over night at 65°C, with embryos incubated in 200 ng/ml (whole-mount) or 30 ng of probe per slide (sections). For sectioning, embryos were equilibrated in 50% sucrose and then embedded in Tissue-Tek O.C.T. compound (Sakura Finetek). Frozen sections were collected at 18 um. For each time-point, embryos were from the same litter and stained for an equal length of time.

Histology

Embryos were dissected in 1 x PBS and fixed for 60 min at 4°C in 10% neutral buffer formalin (Sigma), decapitated, bisected sagittally, the skin removed, and fixed for another 15 min. Embryos were prepared for X-Gal staining as previously described [38]. Whole-mount specimens were further stained with alcizarin red overnight. Sectioned specimens were dehydrated through ethanol series and embedded in paraffin. 10 µM sections were collected and counterstained with nuclear fast red (Vector Laboratories).

For staining for ALP activity, frozen sections were collected at 18 um and stored at –80°C until ready for staining. Slides were brought to room temperature, then washed in acetone, TBST [Tris-buffered saline pH 8.0, 1% Tween-20], and NTMT [0.1 M NaCl, 0.1 M Tris-HCl pH 9.5, 50 mM MgCl2, 0.1% Tween-20] at 4°C, stained with nitro-blue tetrazolium chloride (NBT) and bromo-4-chloro-3-indolylphosphate p-toluidine (BCIP) at room temperature, then counterstained with nuclear fast red. Immunohistochemistry for Phospho-Histone-H3 (Ser10) and Cleaved Caspase-3 (Asp175) was carried out on cryosections. Slides were fixed in neutral buffer formalin, treated with 0.3% H2O2 to quench endogenous peroxidase activity, and blocked with 5% normal goat serum in PBS. Diluted primary antibody (Phospho-Histone-H3 1:200, Cleaved Caspase-3 1:12,900) was applied overnight at 4°C. Sections were developed using a biotinylated rabbit secondary antibody with ABC solution (Vector Laboratories) and DAB.

Supporting Information

Figure S1 Analysis of cell proliferation and apoptosis in the coronal suture. Adjacent sections stained for ALP activity, highlighting the location of the frontal and parietal bones (A–F dotted lines), and antibodies for phospho-histone H3 (A’–C’) or cleaved caspase-3 (D’–F’). Positive cells are marked with arrows. (G) Quantification of the mean number of proliferating cells (y-axis) counted in the suture region (x-axis). (H) Quantification of the mean number of proliferating cells (y-axis) counted in each region of the suture: the frontal bone, parietal bone, and suture mesenchyme (x-axis). The mean number of proliferating cells in each region of the suture was not counted for Gdf6/- embryos since there is no suture mesenchyme and the border between the frontal and parietal bones cannot be distinguished. N = 3 embryos for each genotype and antibody treatment with 5 sections per embryo quantified. Differences the number of proliferating cells per suture region were analyzed using a t-test. (TIF)

Figure S2 Gdf6 expression at E14.5. Gdf6 expression was downregulated in the frontal bone by E14.5 in wild-type (B), Gdf6fl/fl (D), and Gdf6–/– embryos (F). Adjacent sections were stained for ALP to highlight the locations of the frontal and parietal bones (E–G, dotted lines). Previously reported Gdf6 expression in the middle ear bone joints was clearly visible in sections from the same series, acting as a positive control (G,H). (TIF)

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Author Contributions

Conceived and designed the experiments: DEC DPM. Performed the experiments: DEC. Analyzed the data: DEC DPM. Contributed reagents/materials/analysis tools: DEC DPM. Wrote the paper: DEC DPM.

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