Frontal Bone Insufficiency in Gsk3β Mutant Mice

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Abstract

The development of the mammalian skull is a complex process that requires multiple tissue interactions and a balance of growth and differentiation. Disrupting this balance can lead to changes in the shape and size of skull bones, which can have serious clinical implications. For example, insufficient ossification of the bony elements leads to enlarged anterior fontanelles and reduced mechanical protection of the brain. In this report, we find that loss of Gsk3β leads to a fully penetrant reduction of frontal bone size and subsequent enlarged frontal fontanelle. In the absence of Gsk3β the frontal bone primordium undergoes increased cell death and reduced proliferation with a concomitant increase in Fgfr2-IIIc and Twist1 expression. This leads to a smaller condensation and premature differentiation. This phenotype appears to be Wnt-independent and is not rescued by decreasing the genetic dose of β-catenin/Ctnnb1. Taken together, our work defines a novel role for Gsk3β in skull development.

Introduction

The frontal bones develop from neural crest derived mesenchymal cells that initially condense in a position dorsal to the developing eyes. Following the initial condensation, the frontal bones grow by expansion and dorsal migration of the initial cellular condensation [1,2]. These condensations subsequently undergo intramembranous ossification. A number of molecular signals have been implicated in skull growth and patterning, including bone morphogenetic protein (BMP), fibroblast growth factor (FGF), Hedgehog (Hh) and Wnt pathways [3]. In particular, there appears to be a key role for Wnt/β-catenin signaling in the development and ossification of the skull bones [4–7], as well as a requirement for the Wnt inhibitor Axin2 [4,6–10]. However, to date, there have been no reports of a role for glycogen synthase kinase-3 (Gsk3), a key Wnt effector, in the initiation of the frontal bone condensation.

GSK3 is a promiscuous serine/threonine kinase initially identified for its role in glycogen metabolism. In mammals, Gsk3 is encoded by two paralogs, Gsk3α and Gsk3β; each has a unique developmental expression pattern in the skull [11]. During embryogenesis, GSK3 is...
thought to function primarily in the Wnt signaling pathway, where β-catenin degradation is controlled by GSK3 dependent phosphorylation. However, GSK3 has many potential substrates, including GLI proteins, which transduce Hedgehog signaling ([12–14], the insulin receptor IRS-1 and TWIST1 [15,16]. Given the broad function of the two GSK3 proteins, it is surprising that single gene knockouts have minimal phenotypes: the Gsk3α knockout animals are viable [11,17], while Gsk3β knock out animals survive to birth and die due to cleft palate [18–21].

In humans, malformations of the craniofacial skeleton are among the most common congenital anomalies seen in live births. These anomalies include defects in osteogenesis in the skull vault. Premature closure of the cranial sutures fuses the bones of the skull vault together and results in craniosynostosis, while prolonged patency of the sutures results in enlarged fontanelles and unossified regions between the bones in the skull vault. Both of these abnormalities may be caused by disruptions in the intramembranous ossification programme and indeed, key pathways such as BMP and FGF signaling are implicated in disease pathology [22,23]. Several important genes, Msx2 and Twist1, have been directly linked to enlarged fontanelles, but clearly these cannot be the only candidates [22].

Here, we describe the requirements for Gsk3β during development of the neural crest derived frontal bones. In the absence of Gsk3β, the frontal bone primordia are small, leading to enlarged fontanelles. In Gsk3β mutants, we observe reduced proliferation and increased apoptosis at E13.5. Concurrently, the expression of key differentiation markers Fgfr2-IIIc and Twist1 are increased. These data imply premature differentiation of the frontal bones leading to a depletion of the endogenous store of differentiating osteoblasts. Thus, GSK3β appears to be a key regulator of the balance between growth and differentiation in the embryonic skull.

Materials and Methods

Animals

All animals were housed in the New Hunt’s House Biological Services Unit at King’s College London. There are three null alleles of Gsk3β [11,18,20]. We have previously shown that these three lines are allelic and phenotypically identical [11,18]. In brief, all three alleles lead to a loss of function protein. The original allele of Gsk3β (Gsk3βtm1Jrw) is a conventional null with a neomycin cassette replacing the ATP-binding loop [20]. Although this allele was initially reported to be lethal in mid-gestation, further analysis by us and others demonstrate that these animals undergo late gestational or perinatal death [11,18,19,21]. This is confirmed by perinatal lethality in a second null allele (Gsk3βtm1Dgen) which has a lacZ gene replacing the first exon of the protein [11]. Finally, the GSK3β^tm1Grc allele has a protein destabilization domain fused to the 3’ end of the protein which renders it phenotypically null until restored by administration of rapamycin or rapamycin analogues [18,24]. We have verified via western blot that no GSK3β protein is detected in any of the alleles and therefore, all three lines have been used interchangeably in these analyses and, for simplicity, are referred to as Gsk3β^−/−. All conclusions from Gsk3β mutants were based on at least three animals of the same genotype, with comparison to littermate controls. For neural crest lineage tracing, the Wnt-1::cre driver and R26RlacZ reporter lines were used as previously reported [25–27]. In order to generate heterozygous deletions of β-catenin, β-catenin^fl/fl mice were crossed to β-actin::cre driver mice [28,29].

Mouse husbandry

Gestation dates were determined by observation of a vaginal plug, which was designated as embryonic day 0.5 (E0.5). On the indicated days, the pregnant dams were euthanized by CO2 inhalation, or cervical dislocation and the embryos were then collected by caesarian section. All
conclusions were based on a minimum of 3 animals per genotype and the phenotypes that we are reporting here are consistent amongst all of the animals that we analyzed. All animal work was approved by the Ethical Review Board at King’s College London and performed in accordance with United Kingdom Home Office Licenses 70/6607 and 70/7441.

**mRNA in situ hybridization**

Embryos were fixed overnight at 4°C in 4% paraformaldehyde in phosphate buffered saline. Embryos were processed for paraffin embedding and sectioning according to standard protocols, and 10 micron thick sections were mounted on 3-triethoxysilylpropylamine (TESPA) treated Superfrost slides. mRNA in situ hybridization was performed according to standard protocols and revealed with BM Purple [27]. For each probe, control and mutant sections were treated and developed together, and the conclusions were based on at least 3 animals/genotype per gene. The following probes were used: alkaline phosphatase, Cbfa1, Osx1 [30], Fgfr2-IIIc [31], Twist1 [32], Msx1 and Msx2 [33], Gsk3α clone (accession #BC111032, Open Biosystems clone ID 5369444) and Gsk3β (accession #BC006936, Open Biosystems clone ID 2648507).

**Skeletal staining**

Whole mount E18.5 bone and cartilage preparations were performed as previously described [18]. E15.5 skull preparations were fixed overnight in 4% PFA to maintain tissue integrity. These skulls were subsequently stained with alizarin red and cleared in 1% potassium hydroxide. Histological identification of bone and cartilage on sections was performed using traditional picrosirius red/alcian blue staining protocols [34].

**Wholemount lacZ staining**

In the lineage tracing experiments, β-galactosidase activity was visualized by X-gal staining as previously described [27]. Cre negative littermates were used to ensure specificity of staining.

**Cell death and cell proliferation**

Cell death was examined by TUNEL staining on slides using the ApopTag Peroxidase kit (Millipore). Mitotic cells were identified by antibody staining for phospho-histone H3 (PHH3, Cell Signaling) using a standard citrate buffer antigen retrieval and detection with a peroxidase conjugated secondary antibody. To track DNA synthesis, 10 mg/kg bromo-deoxyuridine (BrdU) was administered to pregnant dams by intraperitoneal injection two hours prior to harvesting. Briefly, sections were pre-treated with proteinase K, exonuclease III and DPN1, and BrdU was detected with the anti-BrdU antibody (RPN202; GE Healthcare) [35]. In each case, at least 1 section from 2 animals was counted per genotype. We counted the positive cells in each frontal bone primordia, and because it consists of ordinal data, it cannot be averaged. We found that the data fell naturally into two categories. With the PHH3 data, there were 8 sections that were below 6 positive cells, while the remaining sections were greater than 6 cells. For the TUNEL data, no wildtype section had greater than 3 apoptotic cells, therefore we analyzed the data using these categories.

**Western Blotting**

Tissues were lysed in RIPA, and proteins were separated on a 4–12% NuPAGE Bis-Tris gel with MOPS running buffer (Invitrogen). They were transferred to PVDF membranes and incubated with the activated β-catenin (CTNNB1) antibody (Millipore 8E7), followed by GSK3α/β
antibody (Santa Cruz 0011-A) and HSP-90 (Santa Cruz). The signal was detected using the Millipore Immobilon Western Chemiluminescent HRP substrate detected with X-ray film.

Results

Loss of Gsk3β results in congenital craniofacial anomalies

In the mouse, deletion of both Gsk3 genes is catastrophic, resulting in pre-implantation lethality [36]. This is unsurprising, given the reported ubiquitous expression of both genes [37]. Since then, we have shown that the phenotypes in single knockouts of Gsk3α and Gsk3β are very different, suggesting tissue specific functions of the two genes [11,18]. Gsk3αa is dispensable for life [11,17]. While original knockout of Gsk3β (Gsk3β<sup>tm1Jrw</sup>) appeared to be lethal at mid-gestation stages [20], multiple recent reports have shown that Gsk3β mutants die at birth, from a complete cleft of the secondary palate [11,18,21,36]. However, other associated cranial phenotypes have not been well documented. Thus, we examined the phenotypes of all three Gsk3β null alleles during embryogenesis. As previously noted, all three alleles had a fully penetrant cleft secondary palate [11,18] and data not shown). Externally, the most obvious phenotype was ocular coloboma (Fig 1F). The cranial base was also cleft, with diminished ossification of the presphenoid (PS) (compare Fig 1G to 1B) and a reduction in ossification of the inner ear bones (compare Fig 1H to 1C). Finally, we observed malformations of the skull vault; specifically, the frontal bones are smaller compared to control littermates at E15.5, leading to an enlarged fontanelle (compare Fig 1I, 1J to 1D, 1E).

Gsk3 mRNAs and GSK3 proteins are expressed in the frontal bone primordia

Because of the clear decrease in frontal bone size in Gsk3β mutants, we decided to examine expression of both Gsk3α and Gsk3β in the condensing mesenchyme destined to form the frontal bone. Both GSK3B protein and transcript are expressed in frontal bone primordia at E13.5 (Fig 2A and 2B). Importantly, Gsk3β mRNA expression is absent in the Gsk3β mutant (Fig

![Image](https://example.com/image1.png)

**Fig 1.** Deletion of Gsk3β results in ocular, cranial base and skull vault defects. (A-E) Control Gsk3β<sup>+/+</sup> mice. F-J) GSK3β<sup>−/−</sup> mice. Alizarin red staining marks the bone and alcian blue staining marks the cartilage. (A, F) Loss of Gsk3β results in ocular coloboma (F, arrow). (B, G) At E18.5, in the cranial base, the basioccipital and basisphenoid are cleft and the presphenoid is smaller (arrows, G). (C, H) Ossification of the ear is delayed in the mutant (arrow, H). (D, I) At E15.5 the frontal bone is smaller with a concomitant increase in the width of the metopic suture (m). (E, J) Coronal sections at E15.5. Mutant frontal bones (in J) are smaller than in wildtype (in E). Arrows mark apical extent of frontal bones. bo, basioccipital; bs, basisphenoid; f, frontal; m, metopic; p, parietal; ps, presphenoid.

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Although Gsk3α is also expressed in the E13.5 frontal bone, we found that this expression was unchanged in the Gsk3β mutant animals (Fig 2F).

Neural crest migration follows appropriate paths but is reduced in the Gsk3β-/- mutants

As mentioned above, the frontal bones are formed from neural crest derived mesenchyme [1,38]. Therefore, we considered the possibility that defects in neural crest migration or cell number could lead to a smaller condensation. To test this, we performed a lineage tracing experiment using the neural crest specific driver Wnt1:cre combined with the R26RlacZ reporter [25,26]. We found only subtle changes in the neural crest cells. At e9.5 migration appeared normal, with similar levels of positive cells adjacent to the eye (n = 4/4 mutants; black arrows, Fig 3A and 3B). However, by E13.5, there appears to be a decrease in the β-galactosidase activity in frontal bone condensation in mutant animals (n = 2/2 mutants; blue staining, black arrows, Fig 3D). This suggests that the frontal bone phenotype arises in the condensing neural crest cells after E9.5 and before E13.5.
At E12.5, mutant frontal condensations express appropriate osteoblast markers.

We then considered whether differentiation of osteoblasts was occurring at the right time and in the right place. To do this, we performed mRNA *in situ* hybridization at E12.5, when the condensations are histologically similar in mutants and controls. First, we examined expression of *Cbfa1/Runx2* and *alkaline phosphatase (AP)*. We found that although there was no significant difference in intensity in the *in situ* signals, both *Cbfa1* and *AP* domains are misshapen (n = 3/genotype; Fig 4C and 4D, arrow). The mutant *AP* and *Cbfa1* domains do not extend as far apically and are expanded in the mediolateral domain compared to the wildtype littermate control (Fig 4C and 4D). Both control and mutant frontal bones also express *Msx1* and *Msx2* in appropriate, but smaller domains (data not shown). We concluded that although slightly diminished, mutant bones are undergoing appropriate osteoblastic differentiation at E12.5.

Loss of *Gsk3β* triggers premature differentiation at E13.5

By E13.5, the frontal bone compartments were markedly different between mutant animals and littermate controls (Fig 5). These data suggested that GSK3β is critically important between E12.5 and E13.5. At these stages, both wildtype and mutant condensations continue to express AP in the appropriate domains (Fig 5A and 5F), with some decrease in *Cbfa1* (Fig 5F and 5G). We hypothesized that in the mutant animals, frontal bone osteoblasts might be differentiating prematurely, rather than maintaining a growth and expansion phase. To test this idea, we looked at markers of osteogenic differentiation, *Fgfr2-IIIc* and *Twist1*, by mRNA *in situ* hybridization. We observed that *Fgfr2-IIIc* expression was significantly upregulated in the mutant.
Correlating with the increased Fgfr2-IIIc expression, we also noted a change in the domain of Twist1 expression (Fig 5E and 5J). In the wildtype situation, a stripe of Twist1 expression at the ectocranial border of the frontal condensation distinguishes the frontal bone anlagen from the dermis (arrowheads, Fig 5E). In mutant embryos, we observed that the mesenchyme is not divided into these two compartments; instead, Twist1 expression expands throughout (Fig 5J). From these data we conclude that the subsequent reduced ossification in the mutants is associated with either premature differentiation or aberrant compartmentalization of the frontal bone anlagen.

Changes in Gsk3β mutants are not due to Wnt signaling

Since loss of GSK3β function is predicted to increase the amount of activated β-catenin in the embryo, we tested whether expression of the Wnt targets Osterix-1 (Osx1) and Axin2 was increased in the mutants. Both markers showed no change in the levels of expression in the frontal bone primordia (compare Fig 5C to 5H, and data not shown). We also tested whether the levels of activated β-catenin was changed in the mutants. We found that Gsk3β mutants had no difference in the amount of activated β-catenin at E8.5 embryos or in E18.5 frontal and parietal bones (Fig 6C). Furthermore, heterozygosity of β-catenin did not rescue the wide
fontanelle at E15.5 (compare Fig 6D to 6B). However, the size of the Osx1 domain is much smaller, consistent with the diminished size and shape of the overall condensation.

**Decreased proliferation and increased cell death in the frontal bone condensation**

Finally, we thought that premature differentiation might be accompanied by decreased proliferation. To test this, we examined the number of cells in S-phase by pulsing animals with bromodeoxyuridine (BrdU). We also counted mitotic cells by labeling with a phosphorylated histone H3 (PHH3) antibody. At E13.5 we found a decreased number of cells in S-phase via BrdU staining in the frontal bone (Fig 7E, p<0.05). Surprisingly, we observed an increased number of mitotic cells (Fig 7B and 7F). As GSK3 is known to phosphorylate p27kip1 [39,40], one possibility is that the loss of GSK3β leads to a mild arrest at the G1/S checkpoint. We also considered the possibility that there could be increased cell death in the mutants. Indeed, TUNEL assays revealed more cell death in the mutant frontal bones (Fig 7C). Thus, we observe precocious differentiation and decreased cell numbers in Gsk3β mutants. Taken together, these two mechanisms lead to an overall reduction in the pool of osteoprogenitor cells and a smaller frontal bone.

**Discussion**

Pathological changes in skull development are among the most frequent congenital anomalies associated with live births; thus, calvarial perturbations present a major medical challenge[23].

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**Fig 5. Disorganized frontal bone differentiation in Gsk3β mutants.** mRNA *in situ* hybridization for indicated mRNAs on coronal cross-sections through the condensing frontal bone. (A-E) Gsk3β+/+ animals. (F-J) Gsk3β−/− mutants. (A-B, F-G) The frontal bone condensation expresses AP (F) and Cbfa1/Runx2 (G) in both wildtype and mutant littermates. Mutant condensations (B, G) remain smaller. (C, H) There is no increase in the Wnt dependent osteogenic gene Osx1, which is expressed normally in mutant frontal bone (H). (D, I) Fgfr2-Illic is upregulated in the mutant frontal bone (I, arrow). (E, J) In wildtype animals (E) Twist1 expression marks the ectocranial edge of the frontal bone condensation (arrowheads, E), and borders on an adjacent Twist1-negative region. (J) In mutants, Twist1 is expanded diffusely, leading to an absence of a clearly demarcated, Twist-positive ectocranial border. Scale bar = 100 mm.

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Insufficient cranial bone is frequently attributed to brain abnormalities [41,42]; however, in recent years, it has become clear that mutation in a number of key genes can disrupt the progression of the intrinsic ossification programmes. Here, we demonstrate a requirement for Gsk3β in the initiation and expansion of the frontal bone primordium. We find that Gsk3β mutants display premature osteoblastic differentiation in the frontal bone compartment. This, combined with changes in cell proliferation and increased cell death, leads to smaller frontal bones and a wide fontanelle.

We also considered the possibility that the smaller osteoblastic compartment could arise due to neural crest migration defects. Both Xenopus Twist1 and mammalian Snail1 proteins are reported to be Gsk3 substrates; these are key regulators of neural crest cell migration [16,43–45]. Embryos with a complete loss of Twist1 (Twist1/–) have a severe cranial phenotype, stemming from earlier defects in cranial neural crest migration [46,47]. This is consistent with an evolutionarily conserved role for Twist1 during the critical migratory stages, which then obscures a later role in the ossification and migration of the neural crest derived skull vault mesenchyme. Snail1 mutation also leads to midgestational lethality and thus, roles in the skull are unclear [48,49]. Using lineage tracing to examine migration of the neural crest, we noted no significant changes in the frontal bone-directed cranial neural crest. This may reflect functional redundancy between mammalian Gsk3α and Gsk3β which warrants further study.

Twist1 heterozygotes (Twist1+/–) develop coronal craniosynostosis, owing in part to aberrant migration of Wnt-1cre positive cells into the mesodermal compartment of the coronal suture [50–52]. Coronal synostosis in the Twist1 heterozygotes is thought to result from a
switch of Twist/E2A heterodimers in wildtype animals to Twist homodimers. Twist homodimers preferentially upregulate expression of FGFR2 and subsequent differentiation at the osteogenic front \[53,54\]. In our studies, the loss of compartmentalization of the Twist1 expression domain may also prevent preosteoblasts from migrating and populating the growing osteogenic front. Instead, pre-osteoblasts may differentiate \textit{in situ} in the forming frontal bone anlagen. Though we cannot exclude subtle defects in cell migration, precocious differentiation of osteoblast precursors in the frontal bone primordia will certainly lead to a smaller frontal bone.

The defects we observe in the Gsk3β mutant skulls are more similar to two other mouse models: transgenic dominant negative BMPR1a, and compound Msx2\(^{+/−}\); Twist1\(^{+/−}\) mutants \[22,55\]. Several reports suggest that human BMP receptor 1A mutations also lead to craniofacial dysmorphism \[56,57\], and mutations in human MSX2 lead to persistent calvarial foramina \[58\]. In the mouse, expression of dominant-negative Bmpr1a in the neural crest leads to severe

Fig 7. Loss of Gsk3β leads to decreased proliferation and increased cell death in the frontal bone primordia. (A-D) Coronal cross-sections through the condensing frontal bone, outlined in yellow. (A-B) Mitotic cells were detected by antibody staining detecting phosphorylated histone H3 (pHH3). Mutant sections showed more mitotic cells (B). (C-D) Cell death was detected by TUNEL staining. Mutant sections showed increased cell death (D). (E) BRDU staining revealed a small but significantly lower ratio of cells in S-phase in the mutant frontal bone (p <0.05). (F) Antibody staining for pHH3 positive cells showed more mitotic cells in the mutants. Slides were scored with sections with greater than six (red) or less than six positive cells (blue). (G) All Gsk3β\(^{+/−}\) frontal bone sections had fewer than three apoptotic cells, while the Gsk3β\(^{−/−}\) animals showed increase in cell death, based on the number of cells that have TUNEL-positive staining.

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apoptosis of the frontal bone primordia accompanied by facial clefting [55]. Similarly, enlarged foramina are present in human Saethre-Chotzen patients [59]. In the mouse models for Saethre-Chotzen syndrome (single or compound Mx2-Twist mutants), osteoblastic differentiation markers were reduced by E12.5, while proliferation is not reduced until E14.5 [22]. In contrast, our data suggest a novel etiology for smaller skull vault bone formation, namely premature expression of differentiation markers, and changes in the cell cycle. This implies an acceleration of the ossification programme, leading to a depletion of the osteoblastic progenitor compartment of the frontal bone.

Finally, we considered the possibility that Gsk3β is required for a Wnt/β-catenin dependent function during development of the neural crest derived skull. As we saw no change in Wnt-dependent target genes such as Axin2, and we found no rescue when decreasing the genetic dose of β-catenin (Fig 6), we propose that these functions of Gsk3β are β-catenin-independent. Gsk3α expression may be sufficient to compensate for Gsk3β in Wnt signaling, especially given the critically important roles for Wnt signaling in stem cell maintenance and early development [36]. However, it is worth noting that postnatal deletion of Gsk3β in osteoblasts appears sufficient to increase levels of activated β-catenin [60]. Furthermore, Gsk3β has been reported to phosphorylate and inactivate Cbfa1/Runx2 [21]. Both of these observations could reflect a difference in the prenatal intramembranous ossification programme versus postnatal Wnt-dependent ossification programmes. As the majority of craniofacial congenital anomalies manifest in utero, future studies should focus on distinguishing between temporal and tissue specific substrates of Gsk3.

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Author Contributions

Conceived and designed the experiments: KJL. Performed the experiments: HS-R WY KJL. Analyzed the data: HS-R WY KJL. Contributed reagents/materials/analysis tools: KJL. Wrote the paper: HS-R KJL.

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