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Effects of Thyroxine Exposure on the *Twist 1* +/- Phenotype: A Test of Gene-Environment Interaction Modeling for Craniosynostosis

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Abstract

Craniosynostosis, the premature fusion of one or more of the cranial sutures, is estimated to occur in 1:1800-2500 births. Genetic murine models of craniosynostosis exist, but often imperfectly model human patients. Case, cohort, and surveillance studies have identified excess thyroid hormone as an agent that can either cause or exacerbate human cases of craniosynostosis. Here we investigate the influence of *in utero* and *in vitro* exogenous thyroid hormone exposure on a murine model of craniosynostosis, *Twist 1* +/- . By 15 days post-natal there was evidence of coronal suture fusion in the *Twist 1* +/- model, regardless of exposure. With the exception of craniofacial width, there were no significant effects of exposure; however, the *Twist 1* +/- phenotype was significantly different from the wild type control. *Twist 1* +/- cranial suture cells did not respond to thyroxine treatment as measured by proliferation, osteogenic differentiation, and gene expression of osteogenic markers. However, treatment of these cells did result in modulation of thyroid associated gene expression. Our findings suggest the phenotypic effects of the genetic mutation largely outweighed the effects of thyroxine exposure in the *Twist 1* +/- model. These results highlight difficulty in experimentally modeling gene-environment interactions for craniosynostotic phenotypes.

Keywords

Thyroid Hormone; Cranial Suture; Craniosynostosis; Cephalometrics; Gene Expression

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Introduction

Craniosynostosis is defined as the premature fusion of one or more of the cranial sutures prior to the completion of neural expansion. It is estimated that craniosynostosis occurs in 1:1800-2500 births (Delahyde et al., 2003; Robin, 1999). When it is part of a syndrome, craniosynostosis most often manifests with limb disorders and has strong genetic influence most commonly as part of a cascade of *FGFR* gain of function mutations. However, genomic cases are only a small percentage (10–15%) and most cases of craniosynostosis are classified as non-syndromic with unknown genetic etiology (Johnson and Wilkie, 2011; Morriss-Kay and Wilkie, 2005; Twigg and Wilkie, 2015; Wilkie, 1997; 2000; Wilkie et al., 2007; Wilkie and Wall, 1996). Surgical correction of the fused suture is often implemented to allow for proper neurocranial expansion and follow-up care and surgeries are necessary throughout craniofacial growth adding to both cost and risk associated with craniosynostosis (Parikh et al., 2015; Schaller et al., 2012; Simpson et al., 2016; Zakhary et al., 2014).

Specific genetic (Chen et al., 2003; Crane et al., 2005; Feng et al., 2013; Holmes and Basilico, 2012; Holmes et al., 2009; Morita et al., 2014; Perlyn et al., 2006; Roy et al., 1981; Shukla et al., 2007; Twigg et al., 2009; Wang et al., 2015) and likely non-syndromic animal models (Mooney et al., 1993; Mooney et al., 1998a; Mooney et al., 1998b) of craniosynostosis can be useful in the study of the pathogenesis of premature suture fusion. Current understanding of suture fusion progression has come from molecular differences in patent, fusing, and fused sutures during ontogeny (Coussens et al., 2007; Grova et al., 2012; Lenton et al., 2005; Levi et al., 2012; Rawlins and Opperman, 2008). Although specific to human gene mutations, most genetic murine models of craniosynostosis are not post-natal viable or present with a more aggressive phenotype than that found in human patients (Gong, 2012; Holmes et al., 2009; Motch Perrine et al., 2014; Nah et al., 2012; Twigg et al., 2009; Yeh et al., 2013). Several less severe models do exist and may lend themselves to the study of both growth and development after suture growth disruption, surgical correction, and molecular contribution and/or exacerbation of disorders (Huang et al., 2014; Liu et al., 2007; Mooney et al., 1993; Mooney et al., 1998b; Parsons et al., 2014; Veistinen et al., 2012).

Case, cohort, and surveillance studies have identified agents, environmental or pharmacological, that can either cause or exacerbate human cases of craniosynostosis (Alwan et al., 2007; Browne et al., 2011; Carmichael et al., 2008; Carmichael et al., 2010; Reefhuis et al., 2011). One such agent is aberrant exposure to thyroid hormone (hormone replacement therapy) or exposure to aberrant levels of thyroid hormone during development (maternal thyrotoxicosis) (Ardalan et al., 2012; Carmichael et al., 2015; Chawla et al., 2015; Cohen, 1988; Hashmi et al., 2012; Johnston and Bronsky, 1995; McNab and Ginsberg, 2005; Penfold and Simpson, 1975; Radetti et al., 2002; Rasmussen et al., 2007). Thyroid hormone is known to be important and necessary for limb growth and thyroid hormone receptor influence has been identified in the calvaria of preclinical models (Akita et al., 1996; Akita et al., 1994; O'Shea et al., 2005). However, the link to craniofacial disorders, specifically craniosynostosis, suggests a targeted effect either during a specific time in ontogeny, on a particular developing structure, the undifferentiated suture, or exacerbation of an underlying

genetic defect. Here we investigate the influence of *in utero* and *in vitro* exogenous thyroid hormone exposure on the murine *Twist 1* +/- genetic model of craniosynostosis. The *Twist 1* +/- mutation in humans is linked to single suture synostoses as well as Saethre Chotzen syndrome (Hermann et al., 2012; Robin, 1999; Seto et al., 2007). The murine *Twist 1* +/- model is considered a mild preclinical phenotype exhibiting targeted coronal suture fusion early in ontogeny and some syndactyly. This suture fusion is easily identified, predictable, and has high penetrance allowing for aberration of growth due to environmental factors to be identified (Parsons et al., 2014). We are specifically testing the hypothesis that prenatal thyroxine exposure will exacerbate the craniofacial phenotype of *Twist 1* +/- perinates and alter cellular activity and gene expression of cells garnered from the affected coronal suture.

Materials and Methods

In Utero Exposure

Adult, wild type, C57BL6 control and *Twist 1* +/- (002222) (*Mus musculus*, Jackson Laboratories, Bar Harbor, ME, USA) male and female mice were utilized to produce *in utero* thyroxine exposed and unexposed litters. C57BL6 males were bred with *Twist 1* +/- females or *Twist 1* +/- males were bred with C57BL6 females and separated at ~E13 of pregnancy. At this time, a dose of levothyroxine previously described to cause alteration to circulating thyroid hormone levels (Synthroid, Abbott Laboratories, Abbott, IL, USA) (control dose = no treatment; thyroxine exposed ~415 ng per day) was added to the drinking water provided to pregnant dams (Bowers et al., 1967; Capuco et al., 1999; Krause et al., 2015; Thordarson et al., 1992). Dose was carefully chosen to be less than replacement therapy shown to inhibit Thyroid Stimulating Hormone release, but likely to increase fetal exposure to the exogenous thyroid hormone. Treatment continued until birth of the litters at ~E20. Eleven pn 20 litters were sacrificed for cell isolation. The remaining mouse pups were grown to 15, 20, or 25 days post-natal (pn) when they were sacrificed and whole skulls were fixed with 4% paraformaldehyde for 48 hours and then switched to 70% Ethanol for micro computed tomography (μ CT) analysis. Pn 20 pups were used for cell isolation as this time point allowed for collection of enough tissue to achieve cell isolation and expansion. Post-natal days 15, 20 and 25 were chosen for growth assessment as they offered the ability to employ 2D histomorphometric analysis and 3D μ CT analysis over a range of time when fusion is predicted to occur in this model (Parsons et al., 2014). In addition, at sacrifice tail snips were procured and standard polymerase chain reaction (KAPA Biosystems, Wilmington MA, USA) with agarose gel electrophoresis was performed to confirm *Twist 1* +/- genotypes (Forward: CTTGGGTGGAGAGGCTATTC; Reverse: AGGTGAGATGACAGGAGATC). *Twist 1* -/- is embryonic lethal (Parsons et al., 2014). In order to avoid any uncharacterized craniofacial abnormality of the wild type littermates of *Twist 1* +/- pups, separate wild type C57BL6 crosses were utilized to procure control wild type pups used in growth experiments. Animal Use Protocols were approved by Georgia Regents University Institutional Animal Care and Use Committee (2011-0365), and the Medical University of South Carolina Institutional Animal Care and Use Committee (AR#3341). All breeding procedures were carried out in an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility where all husbandry and related services are provided by the Division of Laboratory Animal

Resources. All procedures and the reporting thereof are in compliance with the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines (Kilkenny et al., 2010).

μ CT

μ CT images were obtained on 15, 20, and 25 day post-natal *Twist 1* +/- mouse pup skulls with a SkyScan 1174 (Kontich, Belgium) at a 22.57 μ m voxel resolution. Scans were obtained on 142 animals (Male=42.8%; Female=43.3%; Undetermined=13.9%) (Table 1). Mouse skulls were reconstructed with NRecon v1.6.4.8 (BrukermicroCT, Kontich, Belgium) as previously described and imported into Amira v5.0 where it was exposed to a Gaussian Smoothing image filter (r50.3 in X, Y, and Z dimensions; isometric kernel size53) to reduce extraneous noise in the images (Parsons et al., 2014). Threshold settings were then set to only visualize bone volume within the skull. Measurements of the length and width of the cranial vault were collected by a single experienced rater (TEP) from each reconstructed mouse skull. Landmarks can be visualized at the following website: http://getahead.psu.edu/viewer.html?id=Adult_Mouse_Skull. Measures were compared at each time point by split-plot ANOVA or Kruskal-Wallis (if data was not normally distributed) where appropriate for effects by dose, $p < 0.05$ was considered significant for post-hoc Bonferonni analyses. Sex of the pups was recorded for future post-hoc investigation but was not considered a factor in the current analyses. All statistical analyses were completed using SPSS 23.0 (IBM, Armonk, NY).

Whole Mount Alizarin Red

A random selection of 3 skulls per group (Wild type, *Twist 1* +/-, *Twist 1* +/- exposed) per time point were used to determine gross dysmorphology and fusion of cranial sutures. Briefly, calvaria were defleshed, dissected, debrained, and placed in 0.5% potassium hydroxide (KOH) for 24 hours. Calvaria were then washed with de-ionized water and placed in 1.6% KOH with 33mg/liter alizarin red (Amresco, Solon, OH, USA) for 24 hours. Skulls were then washed in de-ionized water and placed in a clearing solution (2 parts glycerin, 1 part benzyl alcohol, and 1 part 70% Ethanol) for 3 hours, heated to 45°C for one hour, moved to a final storing solution (1 part glycerin, 1 part 70% Ethanol) and captured using a stereoscope with attached camera (Motic, British Columbia, Canada).

Cell Isolation

After sacrifice at pn 20, 11 litters denoted for cell isolation were separated by sex and genotype and tissue harvested to isolate the native suture cells (Cooper et al., 2010). Soft tissue was removed from the skull and the coronal suture was excised utilizing surgical scissors. The bone pieces were incubated in 5 mL of a 4% collagenase I solution at 37°C on an orbital shaker for 30 minutes, then centrifuged at 5000*g for 5 minutes, and then the supernatant was aspirated. Bone fragments were then resuspended and transferred into 75 mm² flasks with standard culture media, Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Allendale, NJ, USA) containing 10% fetal bovine serum (FBS; Atlanta Biologics, Atlanta, GA, USA), 1% penicillin/streptomycin (penstrep; Lonza), and 0.2% amphotericin B (Lonza) (Cooper et al., 2010). Primary, wild type and *Twist 1* +/- coronal suture cells were cultured in standard media with changes twice a week until 95% confluence was reached. At the time of confluence, cells were seeded at a density of 4,000 cells per well for cell

proliferation and alkaline phosphatase (ALP) assays, and 65,000 cells/well for RNA collection. Cells were treated with standard media only or standard media supplemented with thyroxine. Thyroxine concentrations corresponded to clinical high, normal, and low circulating serum levels (780ng/ml, 78 ng/ml, or 7.8 ng/ml respectively) (Kronenberg and Williams, 2008).

Bioassays

After 3 or 7 days in culture cell viability (proliferation) was assessed with the colorimetric MTS assay (Promega, Madison, WI, USA) (Cory et al., 1991). Briefly, 20 μ L of the MTS solution was added to each well and incubated at 37 °C for 1 hour. The 96 well plate was read at 490 nm on a Gen5 plate reader for absorbance (BioTek, Winooski, VT, USA). Quantitative ALP activity was assessed at 3 and 7 days. Cells were lysed in a 0.1% Triton X lysis buffer and whole cell lysate was measured using SigmaFast p-Nitrophenyl phosphate kit (Sigma-Aldrich, St. Louis, MO, USA) by adding para-nitrophenylphosphate (pNPP) as a substrate assay buffer containing $MgCl_2$ for 30 minutes, and the kinetic of absorbance was read at 405 nm. Standard t-test and ANOVA with post-hoc Bonferonni analyses were conducted on quantitative data (MTS, ALP) where appropriate. Violations of homogeneity of variation resulted in the use of Welch's correction. A total of n=8 wild-type control cell populations were used in the bioassays. For Twist +/- we had n=8 for MTS but some cell population loss for our ALP assay resulting in an n=5 for 3day and n=7 for our 7day Twist +/- data.

qrt-PCR

For RNA expression studies cells were treated with standard proliferation media or a high concentration of thyroxine (780 ng/ml) for 3 or 7 days in culture. Cells were detached, lysates pelleted and RNA isolated using the RNeasy Plus Kit (Qiagen, Valencia, CA, USA). Quantity and quality of RNA was assessed using a Spectrophotometer (Nanodrop 1000, Wilmington, DE, USA). All ratios of absorbance at 260 and 280 nm were greater than 2.0. Experiments were run using a one-step kit for cDNA synthesis and gene expression (TaqMan® RNA-to-Ct™ 1-Step Kit, Life Technologies, Thermo-Fisher Scientific, Walltham, MA, USA) following manufacturer instructions. A master mix was made from nuclease free water, rt-mix, enzyme mix and commercially prepared probe/primer sets (Taqman Gene Expression Assays, Table 2). A total of 50ng of RNA from each sample was added to the master mix for each reaction (run in duplicate) for each gene product for each condition time (3 or 7 day) by treatment (media only control or media supplemented with thyroxine). Data were normalized to 18S ribosomal RNA expression by Δ CT. Quantitative data were compared for gene expression change due to treatment by Δ CT methodology. We used statistical analyses for qrt-PCR data as previously published to determine statistical differences for gene expression (Δ CT and Δ CT standard deviation) after thyroxine treatment for targets of interest at time of exposure (Yuan et al., 2006). Differences were considered statistically significant if p < 0.05. A resulting total of n=4 WT control cell populations and n=9 Twist +/- were used for gene expression studies.

Results

Growth after In Utero Thyroxine Exposure

Figure 1 constitutes micro computed tomography (μ CT) and alizarin red whole mount representatives for 15, 20, and 25 day post-natal control wild-type unexposed, control *Twist 1* +/- unexposed, and *Twist 1* +/- exposed *in utero* to ~415 ng per day of exogenous thyroid hormone. Note the gross dysmorphology including fused sutures of the *Twist 1* +/- animals for both unexposed and thyroxine exposed animals. By 15 days there is clear evidence of coronal suture fusion in the *Twist 1* +/- model, regardless of thyroxine exposure. We examined several parameters of calvaria growth to determine if thyroxine altered the growth trajectory of our *Twist 1* +/- craniosynostotic model. For craniofacial length (Figure 2A), there were significant differences at 15, 20, and 25 days postnatal ($p < 0.001$, $p = 0.020$, $p < 0.001$ respectively). Post-hoc analysis showed wild type control to have greater craniofacial length than both *Twist 1* +/- unexposed ($p < 0.001$) and exposed ($p = 0.027$) at 15 days, *Twist 1* +/- exposed at 20 days ($p = 0.032$), and both *Twist* unexposed ($p = 0.002$) and exposed ($p < 0.001$) at 25 days postnatal. No significant differences in craniofacial length between *Twist 1* +/- unexposed and exposed groups were found at any time point. There was a significant difference at 20 days post-natal ($p = 0.013$) for craniofacial width (Figure 2B). Post hoc analysis showed the *Twist 1* +/- unexposed group to have greater width than *Twist 1* +/- exposed ($p = 0.012$). Cranial capacity (Figure 2C) showed significant differences at 15, 20, and 25 days postnatal ($p = 0.05$, $p < 0.001$, $p < 0.001$ respectively). Post-hoc analyses indicated that wild type controls have greater cranial capacity measures than *Twist 1* +/- unexposed at 15 days postnatal ($p = 0.05$) and *Twist 1* +/- exposed at 20 and 25 days postnatal ($p < 0.001$ for both). Significant differences were found for cranial vault length (Figure 2D) at 15, 20, and 25 days postnatal ($p < 0.001$, $p = 0.004$, $p < 0.001$ respectively). Post-hoc analysis showed wild-type control to have greater cranial length than both *Twist 1* +/- unexposed ($p < 0.001$) and exposed ($p = 0.05$) at 15 days, *Twist 1* +/- exposed at 20 days ($p = 0.001$), and both *Twist 1* +/- unexposed ($p = 0.002$) and exposed ($p < 0.001$) at 25 days postnatal. For cranial vault width (Figure 2E), there were significant differences at 15, 20, and 25 days postnatal ($p = 0.05$, $p = 0.002$, $p < 0.001$ respectively) and post-hoc analysis showed wild-type control to have greater cranial width than *Twist 1* +/- unexposed at 15 days postnatal ($p = 0.028$) and *Twist 1* +/- exposed at 20 ($p = 0.001$) and 25 days ($p = 0.001$) postnatal. Cranial index measures (Figure 2F) showed significant differences at 15 ($p = 0.017$) and 25 days postnatal ($p < 0.001$). Post-hoc analysis showed the *Twist 1* +/- unexposed group to have a larger cranial index than wild type control ($p = 0.023$). Both *Twist 1* +/- unexposed and *Twist 1* +/- exposed had larger cranial indices than wild type control at 25 days postnatal ($p = 0.001$ and $p < 0.001$ respectively). No significant differences in craniofacial length, cranial capacity, cranial vault length, cranial vault width, or cranial index were found between *Twist 1* +/- unexposed and *Twist 1* +/- exposed groups at any time point.

Effects of Exogenous Thyroid Hormone on *Twist 1* +/- Cranial Suture Cells

Figure 3 contains bioassay data for *Twist 1* +/- derived suture cells (and wild type controls for comparison) treated with thyroxine. Data for MTS (proxy for cell proliferation) suggests no statistically significant differences in cell metabolic activity after 3 or 7 days in culture between wild type control, *Twist 1* +/- untreated, or *Twist 1* +/- treated with several

concentrations of thyroxine ($p=0.668$, $p=0.944$). ALP data suggests *Twist 1* +/- cells to have slightly decreased *in vitro* ALP activity under these culture conditions. There was greater overall ALP activity after 7 days compared to 3 days in culture. However, there were no significant differences after 3 or 7 days in culture between wild type control, *Twist 1* +/- untreated, or *Twist 1* +/- treated with several concentrations of thyroxine ($p=0.987$, $p=0.828$).

Investigation of osteogenic gene expression (Figure 4A) (*Runx2*, *Alp*, and *Bglap*) was conducted between wild type control and *Twist 1* +/- untreated and treated cells. Supplemental Table 1 includes data reflecting the endogenous variability of the biological replicate samples. These data suggest the wild type control samples to have less variability than the *Twist* control or treated samples for the osteogenic gene expression. Fold change data for *Runx2* showed slight modulation of this marker between wild type controls and *Twist 1* +/- untreated and treated cells with a slight increase after 3 days in culture (1.3 fold each respectively), and a slight decrease after 7 days in culture (~.85 fold each respectively). There were not however any statistically significant changes for *Runx2*, an early marker of osteogenic commitment, between groups. *Alp* gene expression, was decreased after 3 days in culture for *Twist 1* +/- untreated (.83 fold) compared to wild type control. By 7 days however, an increase in *Alp* gene expression for *Twist 1* +/- cells treated with thyroxine compared to wild type (1.6 fold) was found. *Twist 1* +/- treated cells had greater *Alp* expression at both 3 and 7 days in culture compared to *Twist 1* +/- untreated cells (1.15 and 1.7 fold respectively). Interestingly, *Bglap* (osteocalcin) gene expression showed greater expression for both *Twist 1* +/- untreated (2.6 fold) and treated cells (3.1 fold) compared to wild type controls after 7 days in culture. However, these results were variable, exhibiting great intragroup standard deviations; therefore, no statistically significant differences were observed.

Investigation of thyroid related bone gene expression (Figure 4B, Supplemental Table 1) was conducted between wild type control and *Twist 1* +/- untreated and treated cells Supplemental Table 1 includes data reflecting the endogenous variability of the biological replicate samples. These data suggest the wild type control samples to have similar variability as the *Twist* control or treated samples for thyroid related gene expression, with the exception of *Igf1* and *Ehd1*. Fold change data for *Htra1* shows decreased expression for *Twist 1* +/- untreated compared to wild type control after 3 (.88 fold) and 7 days (.64 fold) in culture. However, it is observed that *Twist 1* +/- treated cells have greater expression compared to wild type control (3 day 1.24 fold, 7 day 1.16 fold) and *Twist 1* +/- untreated cells (3 day 1.41 fold, 7 day 1.82 fold) at both time points in culture. These results were not statistically significant. There was an interesting relationship for *Igf1* gene expression in that wild type control cells had greater gene expression than both the *Twist 1* +/- untreated and treated cells at both time points. After 7 days in culture the difference was statistically significant for *Twist 1* +/- treated cells, $p=0.05$ (0.26 fold or greater than 3 fold downregulation) and trending for *Twist 1* +/- untreated cells, $p=0.06$ (0.29 fold). There were no other statistically significant gene expression alterations for *Igf1*. *Ehd1* expression was observed to be less for *Twist 1* +/- untreated and treated samples compared to wild type controls especially after 7 days in culture (.78 and .74 fold respectively). Data for *Dio3* shows increases in gene expression after 3 days in culture for *Twist 1* +/- untreated (1.47

fold) and *Twist 1* +/- treated (2.34 fold) compared to wild type controls. Expression was observed to be decreased for *Twist 1* +/- unexposed (.60 fold) and normalized to similar levels for *Twist 1* +/- treated cells compare to wild type controls after 7 days in culture. As expected *Twist 1* +/- treated cells had greater gene expression than *Twist 1* +/- untreated cells for both 3 (1.60 fold) and 7 day (1.63 fold) time points, but was not found to be statistically significant. For *Pappa* gene expression, great differences were not observed for wild type, *Twist 1* +/- untreated, or *Twist 1* +/- treated comparisons.

Discussion

Overall our data suggest that the *Twist 1* +/- mutation is a driving factor for aberration in craniofacial growth trajectory compared to controls. We were not able to establish segregating differences in growth parameters and observed few cell based gene activity alterations with physiological challenges of exogenous thyroid hormone. As the activity of cells within the cranial sutures is highly controlled to maintain fibrous tissue between osteogenic fronts allowing for proper craniofacial growth and development, even slight modulations of activity may be biologically important without being statistically significant. In our model of gene- environment interaction some predicted genes were modulated. *Alp* and *Bglap* were slightly modulated by thyroxine treatment and greater in *Twist 1* +/- cells which we assume has a greater contribution of pre-osteoblasts and osteoblasts compared to stem cells or fibroblasts in the cell milieu isolated from the suture compared to wild type controls (Berendsen and Olsen, 2015; Long, 2012; Marini and Blissett, 2013). *Runx2* expression was lower in our *Twist 1* +/- suggesting overall greater contribution of osteoblasts in culture (Berendsen and Olsen, 2015; Long, 2012; Marini and Blissett, 2013). This would make sense as *Twist* is necessary for stemness of cells, particularly within the sutures of the skull (Miraoui and Marie, 2010; Zhao et al., 2015). Heterozygous loss of *Twist* signal, loss of stem cells, and increased osteogenesis are likely responsible for the resulting craniofacial phenotype.

Concerning thyroid related genes contributing to bone phenotype, *Htra1* encodes for a protein that cleaves *Igf* from its receptor allowing for greater local and systemic circulating levels (Spyropoulou et al., 2015; Tiaden and Richards, 2013). This axis is viewed as pro-osteogenic and as thyroid hormone drives increases in these products, it is also viewed to be pro-osteogenic (Motomura and Brent, 1998). Here we see little modulation of *Htra1* *in vitro* and an overall decrease in *Igf* signal in our *Twist 1* +/- cells compared to wild type controls. It is known that osteoblast precursors are particularly susceptible to this axis, showing great increases in both products after *in vitro* administration of thyroxine (Bassett and Williams, 2003; Cray et al., 2013). It is likely that the presence of more mature cells, specifically osteoblasts in these cultures might lead to a lack of responsiveness of *Twist 1* +/- cells in this culture system. Of the three other genes thought to directly modulate thyroid effects on bone or bony development, *Dio3* was observed to have some modulation in this *in vitro* system. This gene is a marker of thyrotoxicosis (Hernandez et al., 2006), which we attempted to model here, and acts to inactivate thyroid hormone in several stages of development. We observe *Dio3* expression highest early in our culture experiments (3 days), but it normalizes later in the time course (7 days). Overall we see no *in vitro* evidence of modulation or exacerbation of the *Twist 1* +/- osteogenic or thyroid related profile.

With no observed *in vivo* or *in vitro* modulation of the *Twist 1* +/- profile after exogenous thyroid challenge there are several limitations but also lessons to be learned from these experiments. There are multiple possible reasons why alterations in phenotype did not occur. *In vivo* dosing or timing of administration may have contributed to the lack of a phenotype. However, the dose is well described in the literature and is in a midrange of physiologically relevant doses that reflect the fluctuation observed during periods of thyrotoxicosis and unlike a more severe thyroid dysregulation such as Maternal Graves Disease (de Lima et al., 1999; McNab and Ginsberg, 2005; Radetti et al., 2002; Rasmussen et al., 2007). The timing of *in utero* exposure was targeted at calvarial development and has been used to elucidate other relevant pharmacological effects on craniofacial development and craniosynostosis (Cray et al., 2014; Durham et al., 2015). In addition, ages chosen for mouse craniofacial comparisons may have led us to miss relevant aberration in growth trajectory. Importantly, growth and morphology of the cranial vault is largely determined by the growth of the brain (Moss and Young, 1960; Riesenfeld, 1967; Sardi et al., 2007; Sun et al., 2004). In this case it is possible that the similarity between wild type and *Twist 1* +/- cells *in vitro* and the dissimilarity between wild-type and *Twist 1* +/- mice *in vivo* is reflective of the cranial sutures following rather than leading craniofacial growth trajectories in this model. Additionally, final form was similar between the *Twist 1* +/- unexposed and exposed animals suggesting overall, the gene was, in this case, too predictive of morphological phenotype and not amenable to challenge with this agent.

In vitro arguments are also founded here. We chose *in vitro* doses relevant to circulating human levels to capture any alteration in cellular activity. None were observed, at least for proliferation and differentiation of paramount importance in craniosynostosis. For gene expression we do observe some indication of increased osteogenic markers in our *Twist 1* +/- samples and even some modulation by thyroid hormone. However, these data were plagued by a great amount of variability between biological samples. This was not an unforeseen result considering the *Twist 1* +/- phenotype presents with patent, fused, and fusing sutures (Miraoui and Marie, 2010; Parsons et al., 2014), compared to our wild type controls which all exhibit coronal suture patency. Thus we might expect to observe more variability within our *Twist 1* +/- samples. In addition, although there were slight aberrations in the thyroid related genes studied here for the *Twist 1* +/- cells, there were no great changes that might be suggestive of a positive or negative effect of the thyroid drug. Again, this may indicate that the suture area including the suture cells do not drive the aberrant craniofacial growth associated with the *Twist 1* +/- model.

In conclusion, we cannot claim a successful modeling of a gene-environment interaction between *Twist 1* +/- craniosynostosis and exogenous thyroid hormone exposure. If anything, in this milder case of a craniosynostotic phenotype, the gene won. There was no additive effect of this potential teratogenic challenge. This is an important point as there is no evidence in the literature that is suggestive of specific interactions between lack of thyroid hormone homeostasis and *Twist 1* +/- driven craniosynostosis. However, from an additive modeling perspective the *Twist 1* +/- murine model provides an available alternative to a non-syndromic craniosynostosis model and has the necessary features for such study (mild phenotype, postnatal viability). Although, this is one sole test of the gene-environment interaction or additivity (Tabery, 2007), other models with more severe phenotypes might be

expected to show even less effect of an “environmental” challenge. It is of potentially more translational impact to study pure environmental challenges for craniosynostosis (i.e. thyroid hormone in the wild type murine system) similar to that which was clinically impactful for other craniofacial disorders (Lammer et al., 1985; Lau and Li, 1995; Yasuda et al., 1986).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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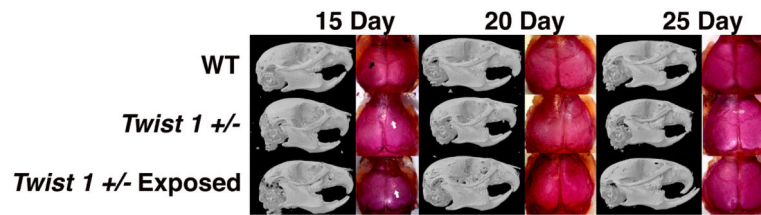


Figure 1. Effects of *in utero* thyroxine exposure on *Twist 1* +/- post-natal craniofacial morphology

Representative μ CT and whole mount staining for pn 15 day (right) 20day (middle) and 25 day (left) for wild type (WT) (top), *Twist 1* +/- unexposed (middle), and *Twist 1* +/- exposed to ~415ng per day thyroxine *in utero* (bottom). Note the dysmorphology including fused coronal sutures in *Twist 1* +/- animals (white arrows) regardless of exposure at all time points. Black arrow indicates patent coronal suture.

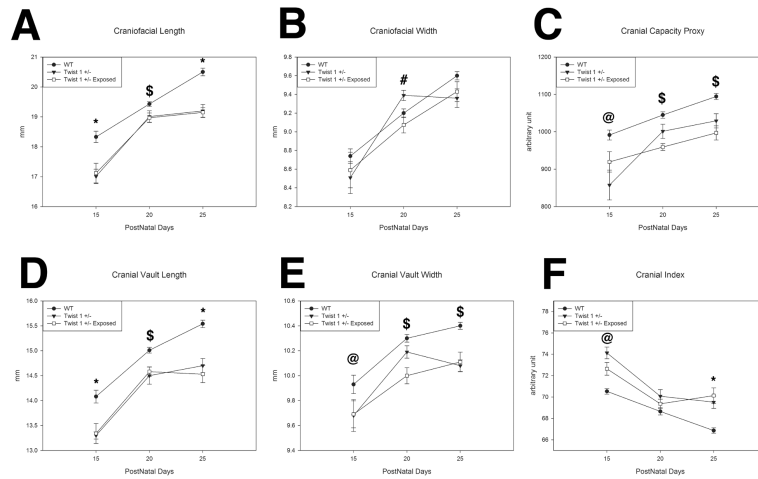


Figure 2. Effects of *in utero* thyroxine exposure *Twist 1* +/- calvarial growth at 15, 20, and 25 days postnatal

A. Wild types have more craniofacial length than *Twist 1* +/- unexposed ($p < 0.001$) and exposed ($p = 0.027$) at 15 days, exposed at 20 days ($p = 0.032$), and both unexposed ($p = 0.002$) and exposed ($p < 0.001$) at 25 days. B. *Twist 1* +/- unexposed have greater craniofacial width than exposed ($p = 0.012$). C. Wild types have greater cranial capacity than unexposed at 15 days ($p = 0.05$), and exposed at 20 and 25 days ($p < 0.001$). D. Wild types have greater cranial length than unexposed ($p < 0.001$) and exposed ($p = 0.05$) at 15 days, exposed at 20 days ($p = 0.001$), and unexposed ($p = 0.002$) and exposed ($p < 0.001$) at 25 days. E. Wild types have greater cranial width than unexposed at 15 days ($p = 0.028$), and exposed at 20 ($p = 0.001$) and 25 days ($p = 0.001$). F. *Twist 1* +/- unexposed have a larger cranial index than wild type ($p = 0.023$), and both unexposed and exposed have larger cranial indices than wild type at 25 days ($p = 0.001$ and $p < 0.001$). (*) Wild type different from unexposed and exposed. (\$) wild type different from exposed. (#) Unexposed is different from exposed. (@) wild type different from unexposed.

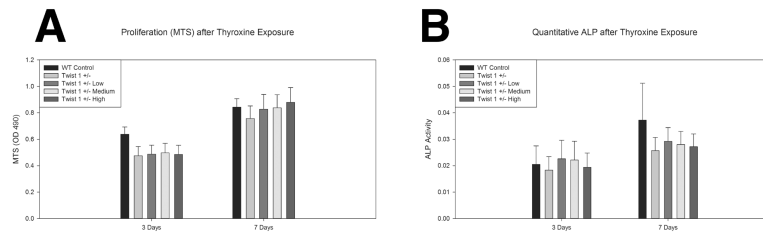


Figure 3. *In vitro* effects of exogenous thyroid hormone on *Twist 1* +/- cranial suture cell metabolism and differentiation

A. Assessment of cell proliferation (MTS) for wild type control, *Twist 1* +/- untreated, and *Twist 1* +/- cranial suture cells treated with low (7.8ng/ml), medium (78ng/ml), and high (780ng/ml) doses of thyroxine for 3 and 7 days demonstrates no significant differences in metabolic activity between groups. B. Assessment of alkaline phosphatase activity (ALP) shows *Twist 1* +/- cells to have slightly decreased ALP activity at 7 days, however, there were no significant differences between groups at either time point.

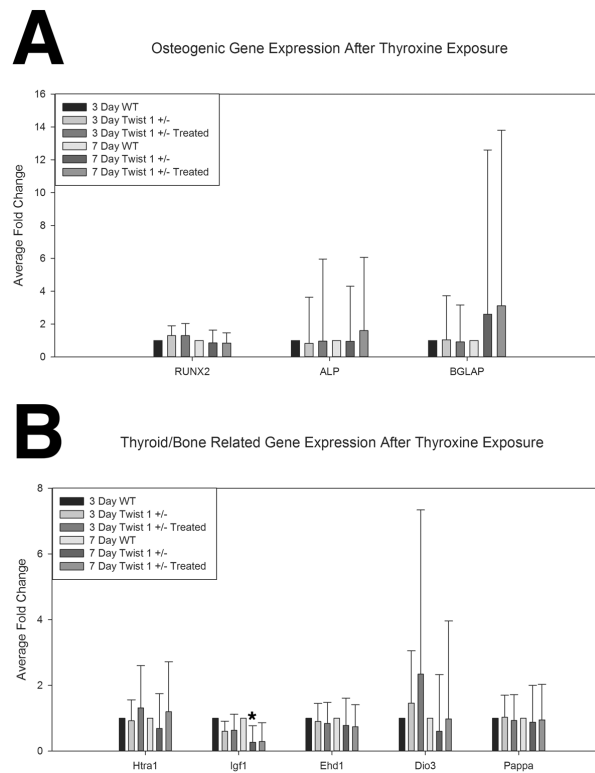


Figure 4. *In vitro* effects of exogenous thyroid hormone on *Twist 1 +/-* cranial suture cell gene expression

A. Osteogenic gene expression from wild type control (n=4), *Twist 1 +/-* untreated (n=9), and *Twist 1 +/-* (n=9) cranial suture cells treated with low (7.8ng/ml), medium (78ng/ml), and high (780ng/ml) doses of thyroxine for 3 and 7 days demonstrated a slight modulation of *Runx2* activity between control and *Twist 1 +/-*, a slight increase in *Alp* associated with treatment, and an increase in *Bglap* (osteocalcin) expression associated with the *Twist 1 +/-* genotype. There were no statistically significant differences in the assessed osteogenic gene markers by treatment, group, or time. B. Thyroid related gene expression for all three groups of cranial suture cells demonstrated a slight effect of treatment for *Htra1* as compared to wild type control and *Twist 1 +/-* untreated, and significantly more *Igf1* expression in wild type control cells (*). Some modulation of *Ehd1*, and *Dio3*, were noted by treatment and genotype, but none was statistically significant. There was no significant change in *Pappa* gene expression noted between groups, or across times. Error bars reflect standard error of the fold change. Wild Type Control Samples measure of dispersion are included in Supplemental Table 1.

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Table 1

Ex Vivo Cephalometric Specimens

Description	15 days PN	20 Days PN	25 Days PN
Wild Type (Twist +/+)	26	19	23
Twist +/- Unexposed	18	13	8
Twist +/- Exposed	8	16	11

Table 2

Quantitative qRT-PCR TaqMan Assay (Applied Biosystems)

Gene Symbol	Gene Name	Assay ID
Runx2	Runt Related Transcription Factor 2	Mm00501584_m1
Alp	Alkaline Phosphatase	Mm00475834_m1
Bglap	Bone Gamma-Carboxylglutamate Protein 3	Mm01741771_g1
Htra1	HtrA Serine Peptidase 1	Mm00479892_m1
Igf1	Insulin Like Growth Factor 1	Mm00439560_m1
Ehd1	EH-Domain Containing 1	Mm01236839_m1
Dio3	Deiodinase, Iodothyronine Type III	Mm00548953_s1
Pappa	Pregnancy-Associated Plasma Protein A	Mm01259244_m1
18S	18S ribosomal RNA	Mm03928990_g1