Biochemical and Biophysical Research Communications 400 (2010) 60–65

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Foxn3 is essential for craniofacial development in mice and a putative candidate involved in human congenital craniofacial defects

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ARTICLE INFO

Article history:
Received 22 July 2010
Available online 5 August 2010

Keywords:
Foxn3
Fork-head
Transcription factor
Mouse model
Craniofacial
Mammalian development

ABSTRACT

The fork-head transcription factors are involved in a variety of physiological processes including development, aging, obesity, and cancer. The fork-head transcription factor FOXN3 has been implicated in cell cycle and transcription regulation at the cellular level. However, the physiological functions of FOXN3 in mammals are not known. To understand the role of the fork-head transcription factor FOXN3 in mammalian development, we have generated a mutant mouse model for the *Foxn3* gene. Our analysis shows that the *Foxn3* mutation leads to partial embryonic and postnatal lethality, growth retardation, eye formation defects, dental anomalies and craniofacial defects. *Foxn3* mutant tissues and cells are also defective in the expression of distinct osteogenic genes. Interestingly, the phenotypes of *Foxn3* mutant mice show a striking overlap with the clinical features of human patients with congenital defects and chromosomal aberrations involving the human *FOXN3* locus. More than three fourths of human congenital disorders involve craniofacial malformations and a majority of the perturbed genetic components that lead to such disorders are yet to be identified. Our results implicate a role for the *FOXN3* gene in the etiology of craniofacial defects in humans.

1. Introduction

The fork-head family is a large family of transcription factors that share a structurally related DNA-binding domain: the fork-head box [1,2]. More than 40 *FOX* genes have been discovered in the human genome. The human fork-head family of proteins is divided into distinct subfamilies (FOXA through FOXQ) and various members have been shown to play diverse roles in development, immune system, cell cycle, and cancer [3]. The FOXO family of transcription factors has been shown to play functional roles in tumor suppression, energy metabolism, and aging [4,5] while the FOXP family members have been implicated in B-cell development, tumorigenesis, and neuronal development [6–8]. At the molecular level, fork-head box proteins bind DNA as monomers and regulate transcription independently or by interacting with transcriptional regulators that can lead to activation or repression of transcription [2,9,10]. In addition, FOX proteins can affect nucleosome positioning and promote gene activation [11,12].

FOXN3 (also known as Checkpoint suppressor 1 and here forth referred to as FOXN3) belongs to the FOXN subfamily [2], and this subfamily consists of five additional members that have diverse physiological functions in mammals. Studies have shown that FOXN1 is a regulator of keratinocyte growth and differentiation of thymic epithelium [13]. The human FOXN2 protein (also known as human T-cell leukemia virus enhancer factor) binds to the human T-cell virus long terminal repeat and is implicated to play a role in transcriptional regulation [14]. The *FOXN4* gene has been shown to be necessary for cardiac and nervous system development whereas *FOXN5* and *FOXN6* have been recently identified as FOXN family members [15–18]. The human *FOXN3* gene has been mapped to 14q32.11, and its C-terminal portion was first isolated as a high-copy suppressor of *Saccharomyces cerevisiae* G1-S and G2-M checkpoint mutants that conferred increased survival of the mutants to DNA damaging agents [19]. The C-terminal portion of the human FOXN3 protein was also found to interact with and inhibit Sin3, a component of the Sin3/Rpd3 histone deacetylase complex (HDAC) in budding yeast [20]. The Sin3/Rpd3 HDAC complex is targeted to specific promoter regions via Sin3 interactions with site-specific DNA-binding proteins [21]. While these heterologous genetic screens suggest a role for FOXN3 in the regulation of

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doi:10.1016/j.bbrc.2010.07.142

gene expression and possibly DNA damage responses, a direct role for the protein in mammalian development is not known due to the dearth of data relating to mammalian models that are defective in *FOXN3* expression. In this study we describe a mutant mouse model that implicates a role for the murine Foxn3 protein in embryonic and craniofacial development.

2. Materials and methods

2.1. Generation of Foxn3 mutant mice

The Baygenomics insertional mutagenesis strategy involves the use of an ATG-less splice-acceptor-beta-galactosidase-neomycin cassette. ES cell clones are then characterized by 5′RACE to identify upstream exons abutting the β-galactosidase-neomycin (βgeo) sequence. One of the ES cell clones that had been characterized to have a genetrap insertion within the *Foxn3* gene was represented in the Baygenomics ES cell library. The *Foxn3* gene trapped ES cells were obtained and further characterized to determine the location of the gene-trap within the *Foxn3* gene. Genomic DNA isolated from ES cells were analyzed by PCR to confirm *Foxn3* disruption by using primers (spanning 1 kb intervals) that were specific for *Foxn3* intron 3 and the gene-trap sequences. Sequencing of the PCR product indicated that the gene-trap was integrated within intron 3 (13,940 bases from the beginning of the intron) of the *Foxn3* gene (Supplementary Fig. 1). At the protein level, the insertion of the gene-trap was determined to be within the Fork-head box domain and upstream of three C-terminal exons consisting of the SIN3 binding domain. The gene-trap strategy employed to generate the *Foxn3* mutant ES cells also leads to the generation of a putative truncated Foxn3-βgeo fusion protein containing the first 218 amino acids and the loss of 239 amino acids at the C-terminus of the wild type protein (Supplementary Fig. 2). The *Foxn3* targeted ES cells were used for blastocyst injections to generate founder mice and subsequent F1 progeny that were backcrossed 3–4 generations into C57BL/6 background. The *Foxn3* mutant and wild type littermates analyzed for the developmental phenotypes were in a mixed inbred C57BL/6-129 background. All protocols and procedures involving the analysis of mutant mice were approved by the University of Tennessee IACUC committee.

2.2. Micro-computed tomography (mCT) image analysis

High-resolution CT images were acquired using a MicroCAT™ II + SPECT instrument (Siemens Medical Solutions, Molecular Imaging, Knoxville, TN). Each image comprised 360 projections at 1 degree intervals and was acquired with X-ray source energy of 80 kVp. CT data were rendered using the Amira 3D image analysis software package (Amira, Version 3.1: Mercury Computer Systems, Chelmsford, MA). At least three animals from each postnatal stage were analyzed.

2.3. Expression analysis of Foxn3 during mouse development

Embryos obtained from timed matings from wild-type females and *Foxn3*$^{+/H}$ males were fixed with 2% paraformaldehyde and stained in a solution containing X-gal (2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.1 M phosphate buffer, pH 7.3). Genotypes of embryos were determined from genomic DNA isolated from yolk sacs.

3. Results

3.1. Foxn3 mutation leads to embryonic and post natal lethality

To understand the role of *Foxn3* in mammalian development, we generated a mutant mouse model using the Baygenomics gene-trap approach [22] (Supplementary Fig. 1). Survival analysis of homozygous mutant animals obtained from multiple heterozygous intercrosses indicated that the homozygous mutants exhibited partial embryonic and postnatal lethality that was lower than the expected Mendelian ratio (Table 1). Chi-squared analysis of the offspring numbers indicated that the differences were significant for the homozygous mutants obtained at weaning (Chi-square; $p < 0.01$). In addition, the fraction of heterozygotes obtained from these intercrosses correlated with the expected Mendelian ratio indicating that the gene-trap did not confer any dominant negative effects in the heterozygotes due to the generation of the truncated *Foxn3-βgal* fusion protein. The partial lethality of the homozygous mutants suggested that the gene-trap might be ineffective leading to the leaky expression of the native *Foxn3* gene. To ascertain the effectiveness of the gene-trap we analyzed the expression of native and mutant *Foxn3* alleles and found that the homozygous mutants expressed low levels of the native *Foxn3* gene indicating the generation of a hypomorphic allele (Supplementary Fig. 2).

3.2. Foxn3 mutation leads to craniofacial defects

To determine the developmental defects of the *Foxn3* homozygous mutants, we analyzed embryos obtained at embryonic stage E13.5 from heterozygous intercrosses and determined *Foxn3* expression patterns in embryos obtained from timed matings between WT and *Foxn3* heterozygotes utilizing the *β-galactosidase-neomycin* fusion gene present within the gene-trap. As shown in Fig. 1A, the homozygous mutant embryos showed abnormal development of the brain, craniofacial regions, and micropthalmia that was consistent with the expression patterns of *Foxn3* during development (Fig. 1B–E). Gross morphological examination of the postnatal homozygous mutants showed severe runting (Supplementary Fig. 3) and the average body weights of the wild type and mutant animals at weaning were 13.5 ± 0.70 g (*Foxn3*$^{+/+}$, $n = 4$), 12.9 ± 0.86 g (*Foxn3*$^{+/H}$, $n = 6$), and 4.75 ± 0.50 g (*Foxn3*$^{H/H}$, $n = 4$). The differences in the body weights between the homozygous mutants and the wild type as well as the heterozygous mutants were statistically significant (Student's *t*-test; $p < 0.0001$; *Foxn3*$^{H/H}$ vs. *Foxn3*$^{+/+}$ and *Foxn3*$^{+/H}$). To further characterize the developmental defects, we performed image analyses of the homozygous mutant and WT littermate controls at postnatal stages P2, P8, and P11 using micro-computed tomography (mCT). A majority of the homozygous mutants showed cranial vault defects of the

Table 1
Embryonic and postnatal lethality of *Foxn3* mutant mice. The genotype analysis of the offspring obtained from heterozygous intercrosses was performed using PCR as shown in supplementary information. The total number of intercrosses (n) analyzed for each developmental stage and the expected numbers of mutant mice based on the numbers of wild-type offspring obtained from intercrosses are indicated within parentheses.

Developmental stage	Total	*WT*	*Foxn3*$^{+/H}$ (expected)	*Foxn3*$^{H/H}$ (expected)	% Survival of *Foxn3*$^{H/H}$
Neonatal day 1 ($n = 9$)	72	21	38 (42)	13 (21)	62
Weanlings at day 21 ($n = 10$)	68	20	42 (40)	6 (20)	30

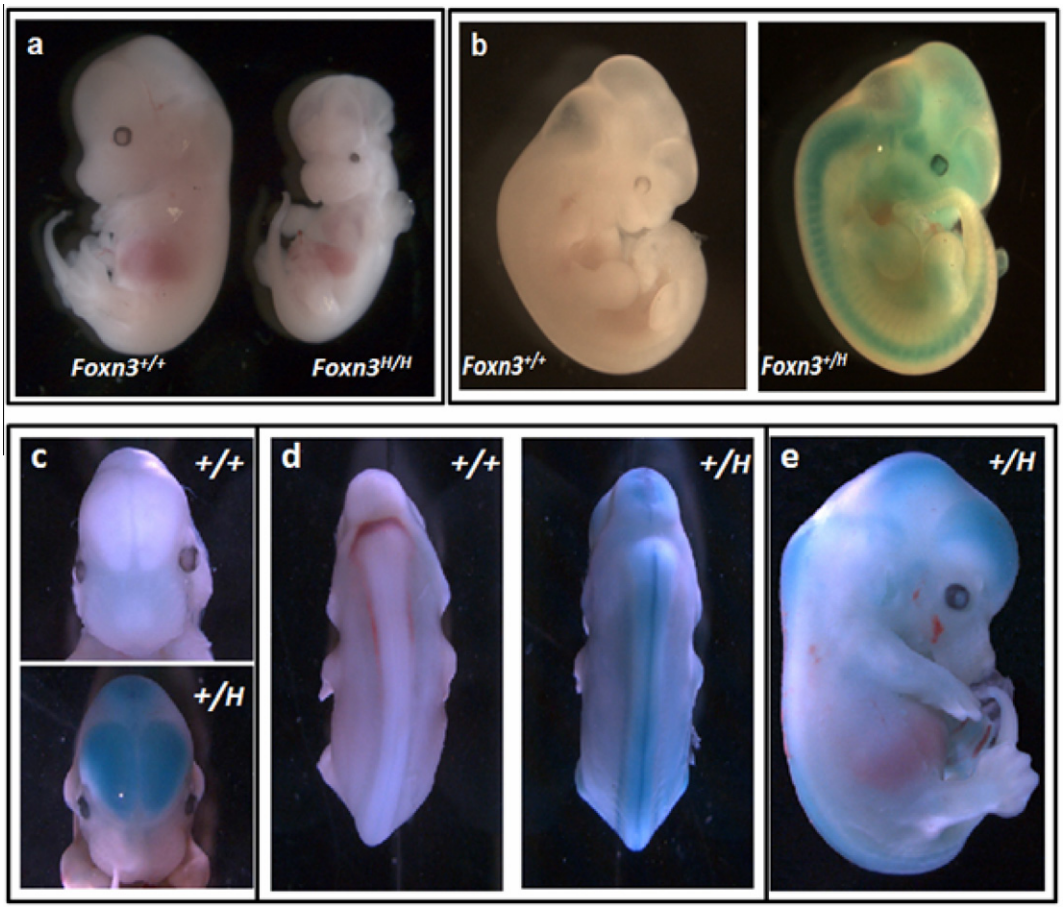

Fig. 1. Analysis of developmental defects in *FoxN3* mutant mice. Panel (A): Embryos obtained at E13.5 from timed heterozygous intercrosses were harvested and photographed. The lateral views of representative embryos photographed at the same magnification are shown. Panels (B–E) Expression analysis of *Foxn3* during embryogenesis. Photographs show representative wild type and *Foxn3* heterozygous embryos at E11.5 (B) and E13.5(C–E) that were treated with X-gal. *Foxn3-β-gal* expression is apparent in the heterozygote [stained blue in panels (B) (right), (C) (bottom), (D) (right) and (E)] and absent in wild-type littermate control [panels B (left), (C) (top) and d (left)]. *Foxn3-β-gal* expression is specific only for the heterozygotes and the wild-type embryos do not show any staining for β-galactosidase. The genotypes of the WT and heterozygotes were determined using yolk sacs of the embryos.

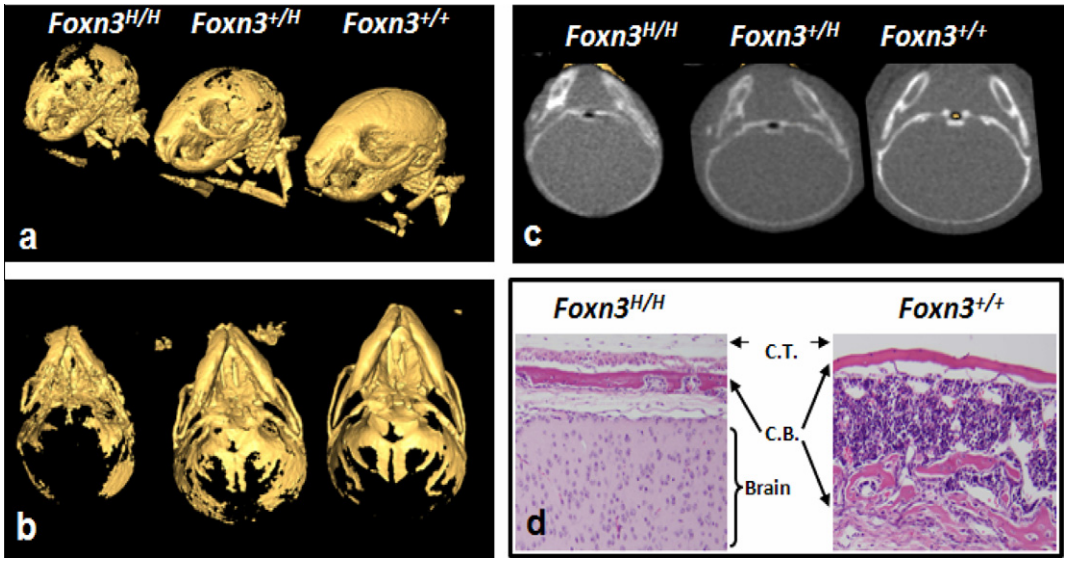

Fig. 2. Characterization of craniofacial defects in *Foxn3* mutant mice. Panels (A and B) Isosurface rendering of micro-computed tomography (mCT) images of the skulls of 8 day old (P8) WT and mutant pups are shown (panel (A) lateral view; panel (B) coronal plane of the dorsal aspect). The mutants have reduced bone structure in the skull bones that appear as hollow structures due to image thresh-holding that was optimized for viewing the calcified structures. Panel (C) Micro-CT scan comparison of P8 WT, heterozygous and homozygous mutant pups (coronal planar view through base of skull). Panel (D) Histological assessment of calvariae in 8 day old $Foxn3^{H/H}$ (left) and $Foxn3^{+/+}$ (right) littermates. Saggital sections of the skull were stained with hematoxylin and eosin and photographed at the same magnification (20×). Notice the calvarium is reduced in thickness in the homozygous mutant (left panel). Identical areas of the same width and length beginning from the connective tissue (CT) of the scalp are shown. CB refers to calvarial bone.

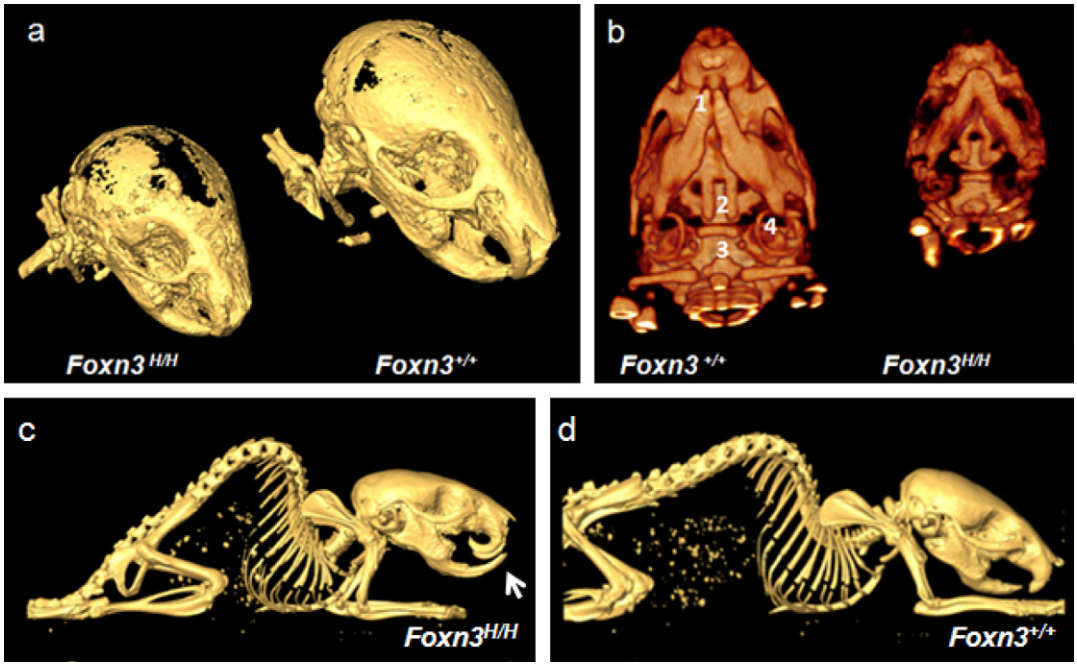

Fig. 3. Craniofacial and dental defects in *Foxn3* mutant mice. Panel (A) Micro-CT scan comparison of 11 day old (P11) wild type (*Foxn3*$^{+/+}$) and homozygous mutant (*Foxn3*$^{H/H}$) littermates. Panel (B) Volume texture rendering of micro-CT images of 2 day old (P2) wild type and homozygous mutant littermate pups shows retarded growth of the mandible which appears to be fused at the midline prematurely. For comparison, the premaxillary area (1), basispehenoid (2), basioccipital (3), and tympanic bones (4) are indicated in the WT animal on the left. Panel (C) and (D): Dental anomalies in adult *Foxn3* homozygous mutant mice. Isosurface rendering of micro-CT images of 6 week old homozygous mutant (C) and WT littermate (D) is shown. The increased growth of incisors in the homozygous mutant is indicated (arrow in panel (C)).

skull, delayed suture closure and defects in the frontal, parietal, and occipital bones of the skull (Fig. 2A and B (P8) and Fig. 3A (P11)). Subsequent histological analysis of calvariae of WT and homozygous mutant littermates indicated a drastic reduction in calvarial bone formation in the *Foxn3* homozygous mutants (Fig. 2D) that was consistent with the mCT-scans that showed a reduced bone density in the homozygous mutants (compare Fig. 2C and D). Examination of the mandible showed reduced size, midline fusion and apparent midline defects indicative of defective development of the basisphenoid, basioccipital, tympanic, and palatal bones in the homozygous mutants at postnatal stage P2 (Fig. 3B). In addition to the craniofacial defects, among the fraction of the surviving homozygous mutants, a high percentage of the mutant mice (~18% in homozygous mutants) also presented dental anomalies with elongated incisors that suggested malocclusion in the homozygotes (Fig. 3C). Interestingly, histological analysis of neonatal and postnatal homozygous animals did not show any defects in the brain tissues (data not shown) despite the high expression of *Foxn3* in the brains of adult animals (Supplementary Fig. 4). Analysis of 3 month old adult homozygous mutant mice indicated an overall growth retardation and scoliosis like phenotype (hunchback spine) in comparison to wild-type littermates (data not shown). To correlate the phenotypes of the *Foxn3* mutant mice with human congenital defects, we performed a comparative literature analysis of the *Foxn3* mutant mouse model phenotypes with the clinical features of human patients with deletions of the human *FOXN3* chromosomal locus (14q31.3–q32.11) [23–27]. Interestingly, there was a striking overlap of the clinical features of human patients and the *Foxn3* mutant mouse model phenotypes (Table 2). While the role of other candidate genes located within the chromosomal deletions described for the human congenital defects cannot be ruled out; the substantial similarities of clinical features of human patients and the *Foxn3* mutant mice underline the importance of *FOXN3* in craniofacial development in mammals.

Table 2
Comparison of phenotypes between human patients with 14q32.11 deletions and *Foxn3* mutant mice. The common phenotypes that involve growth retardation and craniofacial defects between human patients and the *Foxn3* mutant mice are tabulated for the comparative analysis as described in supplementary information section. The numbers of human patients with specific developmental defects are indicated within parentheses. ND, not determined.

Clinical features	14q32.11 deletions	*Foxn3*$^{H/H}$ mice phenotypes
Growth retardation	+(11/12)	+
Microcephaly	+(10/12)	+
Eye deviations	+(11/12)	+/−
Jaw abnormalities	+(10/12)	+
Ear abnormalities	+(8/12)	ND
Dental abnormalities	+/−(5/12)	+/−
Palate abnormalities	+/−(5/12)	+/−

3.3. *Foxn3* regulates the expression of osteogenic genes in the mammalian skull

The craniofacial tissue specific skeletal development defects in the *Foxn3* mutants and its expression patterns suggested that the Foxn3 protein is involved in the transcriptional regulation of genes that are necessary for the differentiation and proliferation of osteoblasts of the mammalian skull. Interestingly, analysis of the promoter regions of a limited number of genes known to be involved in craniofacial development and osteogenesis (*Bmp2, Bmp4, Bpm7,* and *Runx2*) indicated the presence of fork-head domain binding consensus sequences [(A/G)(T/C)AAA(C/T)A] [1,28]. To determine if the expression of the above mentioned osteogenic genes was deregulated in the *Foxn3* mutant animals, we performed RT-PCR analyses using RNA isolated from the skulls of newborn pups using primers specific for *Bmp2, Bmp4, Bmp7,* and *Runx2*. As shown in Fig. 4, there was a reduced expression of the osteogenic genes in the homozygous mutants in comparison to wild-type littermate controls.

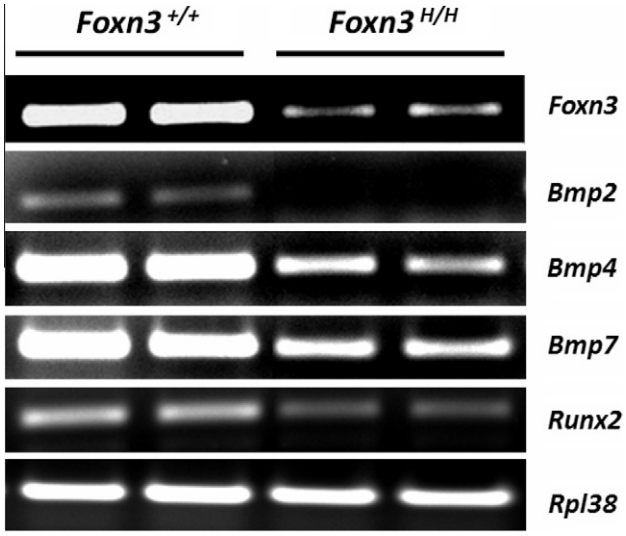

Fig. 4. Reduced expression of osteogenic genes in *Foxn3* mutant mice. Total RNA was isolated from one day old (P1) skulls of wild type and homozygous mutants. RT-PCR analysis was performed with primers specific for the indicated genes and the house-keeping gene encoding the ribosomal protein 38.

4. Discussion

Genetic manipulation of the mouse germline has yielded opportunities to dissect the roles(s) of various gene products in mammalian development and the complexity of gene functions at the organismal level. The recent development of gene-trap based mutagenesis protocols have facilitated and expedited the generation of mutant mouse strains for a large number of genes and allowed the rapid analysis of gene functions in a variety of developmental and physiological pathways [29,30]. In this study we have shown that the *Foxn3* gene is indispensable for mammalian development as the hypomorphic mutants exhibit craniofacial developmental defects that lead to embryonic and postnatal lethality. The craniofacial developmental defects in the *Foxn3* mutants are also correlated by the craniofacial tissue specific expression of *Foxn3* during embryonic development. Furthermore, the phenotypes of the *Foxn3* mutant mice are consistent with recent studies that have shown the involvement of *Foxn3* in craniofacial and eye development in *Xenopus laevis* and suggest evolutionary conservation of Foxn3 functions [31].

The mouse model we have described was generated utilizing the gene-trap strategy and involves the random disruption of genes in mouse embryonic stem cells. Interestingly, our data also indicate that the *Foxn3* gene-trap is not completely efficient in disrupting the expression of *Foxn3* as the homozygous mutant tissues do express the wild-type mRNA albeit at low levels. An inefficient splicing of the upstream exon into the downstream exon (via the circumvention of the splice acceptor present in the gene-trap) has been shown to occur in a minor percentage of gene-trap mutants by our group and others [32,33]. The leaky expression of native *Foxn3* in the homozygous mutants, while explaining the partial lethality phenotypes, provides a novel opportunity to study the role of Foxn3 function(s) at the organismal level that provide data on the role of Foxn3 mediated developmental mechanisms involving heterotypic cell-cell interactions. Such analyses are not possible with knock-out mouse models that suffer from disadvantages relating to embryonic lethality phenotypes or conditional mutants that are not amenable to functional studies involving cell and tissue specific heterotypic interactions during mammalian development.

The spatiotemporal expression of growth and transcription factors and their interactions regulate craniofacial skeletal development in mammals [34]. The mouse skull is composed of frontal, parietal, interparietal, and occipital bones that have specifically evolved to surround and protect the brain. These skeletal components receive lineage contributions from both the cranial neural crest cells and the paraxial mesoderm, both of which migrate to defined locations overlying the brain and subsequently differentiate into osteogenic and chondrogenic mesenchyme between embryonic stages E7.5 and E11.5 [35]. The evolution of neural crest cells has been postulated as the foundation for the initial appearance and evolutionary expansion of vertebrates as it is unique to vertebrates [36]. The skull vault primarily develops by intramembranous ossification, characterized by direct differentiation of the osteogenic mesenchyme into osteoblasts as opposed to endochondral ossification. During skull development, the margins of each bone anlage are populated by highly proliferative osteoprogenitor cells thereby maintaining calvarial expansion [37]. By E15.5 the individual skull bones have acquired their basic structure and are separated by sutures which are composed of fibroblasts and skeletal mesenchyme. Calvarial bone growth and expansion is coordinated by the growth of the brain through continued production of the osteoprogenitors present within the suture [37]. Several growth factors and transcription factors have been implicated in craniofacial and skeletal development [38,39]. The reduced expression of the osteogenic genes in the craniofacial tissues of the *Foxn3* homozygous mutants suggests that the Foxn3 protein regulates the expression of genes necessary for osteogenesis and plays a regulatory role in the craniofacial skeletal development. However, the molecular mechanism(s) of the aberrant expression of osteogenic genes in the *Foxn3* mutants remain to be ascertained. Interestingly, the FOXN3 protein has been shown to associate with Sin3, a component of the Sin3/Rpd3 histone deacetylase complex (HDAC) in budding yeast and inhibit the activity of Sin3/Rpd3 HDAC complex [20]. The Sin3 complex is targeted to specific promoter regions via Sin3 interactions with site-specific DNA-binding proteins [21]. Our data suggests that the loss of osteogenic gene expression in the *Foxn3* mutants is potentially due to the loss of inhibition of Sin3 complex that can in turn lead to deacetylation of histones within the promoters of the osteogenic genes resulting in their repression. Nonetheless, a direct role for Foxn3 in the transcriptional activation of specific genes necessary for craniofacial development cannot be ruled as the interaction studies have utilized heterologous systems to determine the interactions between FOXN3 and Sin3 [20].

5. Conclusion

Our results show that the Foxn3 protein is in involved in the regulation of craniofacial development in mammals and affects the expression of distinct osteogenic genes. The substantial overlap of the phenotypes of the *Foxn*3 mutant mouse model with the clinical features of human patients with deletion of the *FOXN3* locus highlight its importance in the etiology of human craniofacial defects and provide a potential diagnostic tool in their assessment. Our studies could possibly pave way for therapies aimed at modulating the expression of osteogenic genes in the treatment of craniofacial disorders in the future.

Acknowledgments

This work was supported by the Physicians Medical Education and Research Foundation and start-up funds from the UTK BCMB department.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.07.142.

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