USING RNAi TECHNOLOGY TO
DOWN-REGULATE SIX2 EXPRESSION IN VITRO

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ABSTRACT

The Brachyrrhine (Br) mutant mouse, which displays frontonasal dysplasia and renal hypoplasia, has previously been described. Linkage analysis mapped the semi-dominant Br mutation close to the homeobox transcription factor Six2, which is normally expressed in the facial and metanephric mesenchyme during embryonic development. The purpose of this study is to evaluate the role of Six2 in craniofacial and renal branching morphogenesis by quantifying its expression level in the facial prominences and using immunohistochemistry to visualize branching of the ureteric bud in Br mice. In addition, an in vitro system utilizing RNA interference (RNAi) technology was developed to determine whether Six2 could be experimentally down-regulated. Medial nasal (MNP), lateral nasal (LNP) and maxillary prominences (MAX) of E11.5 embryos were dissected and Six2 expression was measured with qRT-PCR. Six2 expression was highest in the MNP, with about 2-fold less in the MAX, and 3-fold less in the LNP of wild-type embryos. Our data indicate a haploinsufficient pattern of Six2 expression in each of the three sets of facial prominences. In addition, kidney organ explants were dissected from E13.5 mouse embryos and immunostained to reveal differences in ureteric bud branching patterns between the three genotypes (+/+), Br/+ and Br/Br). We confirmed a down-regulation of Six2 in a cell culture system utilizing five RNAi constructs. These data indicate a lack of Six2 expression may play a role in the development of a median facial cleft and its reduced effect in the kidney may lead to renal hypoplasia. Additionally, an in vitro system was established that will allow experimental down-regulation of Six2 to assess effects on morphogenesis.
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INTRODUCTION

The development of the mammalian primary palate is crucial for the natural formation of the mid-face. Malformations of this structure result in catastrophic results for the fetus, including difficulty in breathing, suckling, mastication and speech formation. Frontonasal dysplasia (FND) was explained as an umbrella term for two or more of the following: (1) ocular hypertelorism, (2) broadening of the nasal root, (3) median facial cleft affecting the nose and/or upper lip and palate, (4) unilateral or bilateral clefting of the alae nasi, (5) lack of formation of the nasal tip, (6) anterior cranium bifidum occultum and (7) a V-shaped or widow’s peak frontal hairline (DeMyer, 1967; Sedano et al., 1970; Sedano and Gorlin, 1988). FND has an occurrence of 0.43-0.73% in the human cleft population (Apesos and Anigian, 1993).

Roizenblatt et al. (1979) has shown FND can be associated with renal hypoplasia (RH), a developmental disturbance resulting in an abnormally small kidney. Renal hypoplasia is described as an insufficient number of nephrons secondary to defective branching of the ureteric bud during renal morphogenesis (Glassberg, 2002). Renal failure results due to inadequate filtering, improper excretion of wastes, failure to retain electrolytes and inability to properly concentrate urine.

Development of the face

The mid-face develops as a result two processes: (1) the fusion of three sets of facial prominences: the medial nasal prominences (MNP), lateral nasal prominences (LNP) and the maxillary prominences (MAX) to form the future primary palate, consisting of the
upper lip, alveolus and the anterior hard palate, and (2) the merging of the bilateral MNP to form the philtrum. The secondary palate, derived from a different embryologic origin and responsible for the development of the hard palate posterior to the incisive foramen and soft palate, forms later.

The neuroectodermally derived trigeminal neural crest mesenchyme from the midbrain and the first two rhombomeres migrates into the upper jaw primordia where the mesenchyme proliferates to form the orofacial prominences (Johnston, 1964, 1966; Lumsden et al., 1991; Schilling and Kimmel, 1994; Rossel and Capecchi, 1999). The high rate of proliferation of the neural crest mesenchyme is maintained by an interaction at the epithelial-mesenchymal interface (Minkoff and Kuntz, 1977, 1978). This interaction, mediated by developmental factors, is significant for the sustained growth of the facial primordia (Minkoff, 1991).

The morphogenesis of the human primary palate begins at approximately 41 days post-fertilization (O’Rahilly, 1978) and in the mouse at 10 days and 18 hours postfertilization (Reed, 1933; Trasler, 1968). The basis of the primary palate, the upper lip, forms from the MNP (Diewert and Lozanoff, 1993). The bilateral MNP merge at the facial midline to form the central tuberculum of the upper lip (Diewert and Shiota, 1990; Diewert et al., 1993a; Diewert and Lozanoff, 1993; Rude et al., 1994). The continued growth and migration of the mesenchyme underlying the epithelium of the MNP eliminates the distance between the paired MNP, distending the epithelium and merging the two structures (Patten, 1961). Completion of the sides of the lip and closure of the palate requires the fusion of the MNP with the MAX at the nasal fin and the breakdown,
by apoptosis, of the epithelial seam to achieve mesenchymal confluence and provide continuity of the upper lip (Shuler, 1995). This completion of the lip also separates the nasal pit from the stomodeum.

*Signal transduction pathways associated with facial development*

The differentiation and growth of the mesenchymal cells composing the facial primordia and the closure of the primary palate are under the control of spatial and temporal signal transduction pathways. Growth factors directly stimulate cellular differentiation, proliferation and migration. Bmp4, Bmp2 and Bmp7 appear to be involved in early head development by inducing and determining the migration patterns of the neural crest mesenchyme as well as their condensation and proliferation in the facial primordia (Nie et al., 2006). Bmp2 and Bmp4 have been suggested to be required for the migration of the cranial neural crest cells to the facial primordia (Kanzler et al., 2000). Bmp4 and Bmp7 are expressed in epithelium of the facial primordia and are associated with the expression of Bmp2 in the mesenchyme (Barlow and Francis-West, 1997, Bennett et al., 1995, Francis-West et al., 1998). Later, Bmp4 is also expressed in the mesenchyme of the facial primordia (Barlow and Francis-West, 1997). Altered expression of this signaling cascade leads to irregular development of the facial primordia (Barlow and Francis-West, 1997). The *Fgf* gene family, including *Fgf1*, *Fgf2*, *Fgf4*, *Fgf5*, *Fgf8*, *Fgf9* and *Fgf12*, are also expressed in the facial primordia, although their expression patterns are not well understood (Francis-West et al., 1998; Colvin et al., 1999). However, Richman and Crosby (1990) found bFGF is involved in the
development of the facial primordia by regulating the enlargement and stimulating an increase in proliferation in the frontonasal mass mesenchyme.

The expression of TGF-β family of genes is pivotal for palatogenesis via a receptor signaling complex, the Alk-5/Smad pathway. TGF-β3 has been suggested to be heavily involved in palatal fusion, such that its expression is responsible for the disappearance of the midline epithelial seam (Dudas et al., 2004). This disappearance can be achieved through three different means: apoptosis, cell migration and epithelial-to-mesenchymal transdifferentiation (Martinez-Alvarez et al., 2000).

These signal transduction pathways are under the control of specific transcription factors. The Hox family of genes, a particular collection of homeobox genes, functions heavily in patterning the body axis, however, Hox genes also mediate craniofacial developmental patterns in both mice and humans (McGinnis et al., 1984; Krumlauf, 1993; Vieille-Grosjean et al., 1997). Hox7, Hox8 and Hox9 are expressed in several areas where epithelial-mesenchymal interactions occur during embryogenesis (MacKenzie et al., 1991a, 1991b). Their expression patterns form gradients, identified by DeRobertis et al. (1991), suggesting they provide positional information through signals that influence morphogenesis where epithelial-mesenchymal interactions occur.

Development of the kidney

The development of the kidney begins with the appearance of the primary nephric duct on day 22 in humans and day 8 in mice (Gilbert, 2000). The ureteric bud (UB), an outgrowth of the primary nephric duct, extends into the adjacent metanephric
mesenchyme (MM) where the invading bud epithelia begin branching morphogenesis. Upon branching, MM cells condense around the tips of the branching UB before undergoing a mesenchymal-to-epithelial transition, beginning nephrogenesis. Each cluster of epithelial cells elongates into a “comma” shape followed by a characteristic S-shaped tube.

Differentiation of the epithelial cells ensues to form the functional cells of the mature nephron: capsule cells, podocytes and distal and proximal tubule cells. During differentiation, a continuous lumen is formed between the UB, destined to become the renal collecting ducts, and the newly formed nephron by the breakdown of the basal lamina between the two structures, allowing material to pass from the nephron to the collecting duct (Bard et al., 2001).

*Signal transduction pathways associated with kidney development*

The embryonic kidney develops as the result of reciprocal induction from the condensing MM and the UB. As described by Saxén (1970) and Sariola (1982), if the MM is induced by other tissues, such as embryonic salivary gland or neural tube tissue, the MM responds by forming only kidney tubules and no other structures. GDNF, synthesized in the condensing MM, causes the UB to branch from the primary nephric duct. GDNF binds to and activates a receptor complex consisting of Ret receptor tyrosine kinase and GDNF family receptor α1 (GFRα1) (Airaksinen and Saarma, 2002). The GDNF receptor complex is synthesized in the primary nephric duct and becomes concentrated in the UB (Schuchardt et al., 1996). GDNF also induces secondary buds
once the UB has entered the MM (Sainio et al., 1997). Gdnf−/− mice demonstrated renal agenesis and died shortly after birth, as did mice deficient for the GDNF receptor (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996; Gilbert, 2000).

The UB induces the MM to congregate at its tips and differentiate to generate the functional cells of the nephron. Grobstein (1955) and Koseki et al. (1992) showed if the MM is not induced by the UB, the mesenchymal cells undergo apoptosis. The signals from the UB resulting in induction of the MM include FGF2 and BMP7. Both factors inhibit apoptosis and promote the condensation of MM cells around the tips of the branching UB (Perantoni et al., 1995; Dudley et al., 1995; Luo et al., 1995).

The transcription factor Pax2 indirectly promotes the mesenchymal-to-epithelial transition by upregulating the expression of Ret as part of the GDNF receptor complex on the UB epithelium. It is this epithelium that induces the condensation and differentiation of the MM. In Pax2−/− mice, Ret loses expression in the nephric duct by E10.5 and ureteric buds capable of inducing the MM do not form, resulting in renal agenesis (Torres et al., 1995). Hence, Pax2 is an important factor necessary for establishing and maintaining the nephrogenic zone.

Six2, a transcription factor expressed in the nephrogenic zone MM, is required to suppress the mesenchymal-to-epithelial transition initiated by the UB in order to maintain an adequate number of progenitors. The failure to renew the mesenchymal cells responsible for nephrogenesis results in severe renal hypoplasia (Self et al., 2006). Six2 appears to work in conjunction with Eya proteins, which localize to the nucleus and may act as a coactivator and up-regulator of Six2 transcription (Pignoni et al., 1997; Ohto et
al., 1999; Zou et al., 2004; Purcell et al., 2005). Gong et al. (2007) identified a Six2 promoter binding site crucial for this activation and proposed a Hox11-Eya1-Pax2 network that translates anterior-posterior positional information within the embryonic kidney. Brodbeck et al. (2004) showed Six2 possesses a transcriptional activation domain in the C-terminus and may activate various kidney morphogenetic factors, such as GDNF. Thus, Six2 appears to play an important role in nephron development.

The Br mouse as a model for abnormal facial and kidney development

A mouse mutant on the 3H1 background strain with frontonasal dysplasia has been identified (Lozanoff, 1993). This phenotype, associated with the semidominant Brachyrrhine (Br) mutation, was induced during the testing of irradiation effects on chromosome structure. The adult Br mouse displays a severe median facial cleft, as described by DeMyer (1967), resulting from the failure of the MNP, LNP and MAX to fuse. Linkage analysis showed that the likely candidate involved in the mutation is Six2, located on distal chromosome 17 (Table 1; Fogelgren et al., unpublished data). Additionally, the Br mouse exhibits renal hypoplasia. This type of malformation is coupled with a decrease in Six2 expression in the differentiating metanephric mesenchyme.

The long-term goal of this research is to determine the role of Six2 in embryonic development using the Br mouse model. To achieve this goal, we will first examine the expression of Six2 in embryonic tissues. The first specific aim is to characterize the expression of Six2 in the facial prominences in wild-type (+/+), heterozygous (Br/+), and
Table 1. Microsatellite recombination data. Br mutation mapping data using microsatellite markers along mouse chromosome 17. Shown are both Cast and Balb backcrosses, where X is number of recombinants among N total backcrossed mice analyzed. The calculated distance (in centimorgans) and LOD scores are shown on the right. Permission to present data kindly provided by Fogelgren et al. (unpublished data).

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homozygous mutant (Br/Br) embryos. Previous work in our laboratory showed that Six2 expression is reduced in the cranium of mutant mouse embryos and this work will build upon the previous findings by examining expression in individual facial prominences. We expect that expression will be reduced most in the MNP of Br/Br embryos since these mice show median facial clefts, with intermediate expression in Br/+ mice, and greatest expression in +/+ animals. The significance of this work is that it will show whether Six2 expression is associated with specific facial prominences and
median facial clefts. At the same time, it will provide justification for establishing an *in vitro* system for experimental testing of *Six2* function in embryonic facial tissues.

The second specific aim is to determine whether the UB branching pattern in the mutant embryos is reduced using an *in vitro* system. Previous work in our laboratory has already shown conclusively that *Six2* expression is reduced in the kidney of mutant embryos. Thus, we expect that branching will be reduced in *Br/+* mice and severely reduced in the *Br/Br* condition when compared to the wild-type (WT) kidney. The significance of the current work will show whether this reduced expression is associated with decreased UB branching and establish a whole organ kidney culture system as an experimental basis for future work assessing the effects of *Six2* down-regulation on UB branching.

The third specific aim is to apply RNA interference (RNAi) technology to determine whether *Six2* expression can be reduced in an *in vitro* system. This objective will be accomplished by determining whether *Six2* is expressed in an established cell line using qRT-PCR methodology. If present, RNAi, including *Six2* shRNA transfection into the cell line, will be applied with the expectation that *Six2* expression will be reduced. If so, then this work will establish a method to decrease *Six2* transcription in an *in vitro* system. The significance of this work is that it will lay the foundation for future experiments to determine the effects of *Six2* on embryonic tissues *in vitro*. 
MATERIALS AND METHODS

Animals

All procedures were carried out in accordance with IACUC specifications and were approved by the Laboratory Animal Services, University of Hawai‘i. Adult 3H1 mice were housed under standard conditions with a 12-hr light cycle and supplied with tap water and Purina Mouse Chow ad libitum. Embryos were obtained via reciprocal crosses of *Br* adults. Females were examined for a vaginal plug; if present, the day was designated E0.5. Embryos were obtained on day E11.5 for analyses regarding facial development, since this is the critical point during which the MNP merger occurs. Kidneys were collected at E13.5, since this is the developmental period when the ureteric bud interacts with the metanephric mesenchyme and has begun its initial subdivisions. All were staged using Theiler criteria (TS) ensuring the developmental stage of each embryo was similar to the conception day (E) designation (Theiler, 1989). Only animals of the same E designation and TS were compared. At the appropriate embryonic stage, the gestational female was anesthetized with an isoflurane inhalant, cervical dislocation performed and embryos collected via Caesarian section.

Genotyping

Previous physical mapping analysis showed the *Br* mutation is located in an approximately 171 kb region of murine chromosome 17 that includes only one known gene; namely *Six2*. Microsatellites were tested for recombination to establish a primer suitable for genotyping (Fogelgren et al., unpublished data). To generate animals that
could be genotyped successfully, 3H1 Br/+ mice were outbred with inbred lines of Castaneous (3H1 Br/+ x Cast) and Balb (3H1 Br/+ x Balb) mice. DNA samples were obtained from each embryo using extraneous tissue after the kidneys or facial prominences were dissected free from surrounding structures. Genotyping was conducted using primers to amplify D17Mit76 (D17Mit76-f: 5'-AGC AAA GCT TAG TGT TIC OC-3'; and D17Mit76-r: 5'-GGG GAT GCA AGT TAC TCC TC-3'). All primers were synthesized at the University of Hawai'i Biotech Core, Honolulu, HI, UH core facility. Pairs of oligonucleotides were amplified using a Thermo Electron thermocycler with a PCR profile consisting of an initial denaturation at 94°C for 4 minutes, then 35 cycles of 30 seconds at 94°C (denaturation), 30 seconds at 55-60°C (annealing), and 30 seconds at 72°C (extension), with a final extension at 72°C for 4 minutes. The PCR products were separated by electrophoresis in 4% Metaphor agarose gels and stained with ethidium bromide. The gels were photographed with a Kodak Gel Logic 200 photographic module. Each gel included a 25 bp size ladder, blank water control, control 3H1, control Balb or Cast and the embryos for analysis.

Genotyping was based on the number of amplimers present. An embryo that displayed only one 3H1 amplimer was scored as a homozygous mutant (Br/Br) since it only possessed the 3H1 resulting from the outcross. If two amplimers were present, it was identified as a heterozygous mutant (Br/+) since it possessed both a 3H1 and outcross allele for D17Mit76 (one 3H1 and one of either Balb or Cast). If one amplimer consistent with the Balb or Cast allele was present, the sample was identified as a homozygous WT animal (+/+).
qRT-PCR of Six2 in facial prominences of WT and Br mice

Previous work in our laboratory demonstrated the kidney in Br mice demonstrates a haploinsufficient expression pattern of Six2 utilizing the quantitative real-time polymerase chain reaction (qRT-PCR). However, the Br mutant mouse strain had not been tested for Six2 expression in the facial prominences. Thus, we undertook a qRT-PCR analysis of the facial prominences to determine whether Six2 is expressed in a haploinsufficient pattern in the facial prominences of homozygous mutant, heterozygous and homozygous normal 3H1 x Balb and 3H1 x Cast mice.

Dissection of the facial prominences for mRNA extraction was carried out at E11.5, as this is the stage at which the MNP merger occurs and contact is established between the MNP and MAX, beginning the continuity of the upper lip (Wyszynski, 2002). The paired MNP, LNP and MAX were dissected using microforceps and placed in RNA later (Ambion) until genotypes could be confirmed. The dissection was accomplished by first staging the embryo, placing it laterally, and then removing the MAX. The embryo was then placed in the frontal position and the MNP and LNP were separated from the remaining cranium. The nasal pits were then transected at the superior and inferior points separating the LNP from the MNP. After each of the prominences were dissected away from the surrounding structures, any extraneous tissue seen still adhering to the prominence edges was removed. This ensured mRNA was extracted only from prominence tissue and not surrounding structures. Each pair of facial prominences were placed immediately and individually in 400 μL of RNA later and stored at 4°C for one to three weeks before processing. A total of 22 embryos from 4 litters derived from
reciprocal 3H1 x Balb Br/+ or 3H1 x Cast Br/+ crosses were collected. Each litter contained +/-, Br/+ and Br/Br embryos. A separate set of litters (2) were obtained from stock 3H1, Balb or Cast matings to provide additional (12) control embryos.

mRNA from individual pairs of facial prominences was extracted using Qiagen’s RNeasy Mini Kit according to the included protocol for animal tissues. Total RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) and the included protocol. qRT-PCR reactions (25 μL final volume) were performed in triple replicates with 1 μL of cDNA, 1 μL of each primer and 12.5 μL of Bio-Rad’s IQ SYBR Green Supermix with the MyiQ iCycler thermocycler and single color real-time PCR detection system (Bio-Rad). Primers to amplify Six2 (Six2-f: 5’-CTC ACC ACC ACG CAA GTC AGC AAC-3’; and Six2-r: 5’-CAC CGA CTT GCC ACT GCC ATT GAG-3’) and the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH; GAPDH-f: 5’-TGC ACC ACC ACC TGC TTA GC-3’; and GAPDH-r: 5’-G GC ATG GAC TGT GGT CAT GAG-3’) were used. The thermocycle profile used was an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 15 sec (denaturation), 59°C for 30 sec (annealing), 72°C for 60 sec (extension) and 80°C for 6 sec (quantification of fluorescence). Product-specific amplification of Six2 and GAPDH was confirmed by melting curve analysis. The threshold cycle, C(t), was established at the linear portion of the log scale curve and the ratio of Six2 to GAPDH was calculated using the 2−ΔΔC(t) method (Livak and Schmittgen, 2001).
Kidney organ explants

The uterus was removed and placed in PBS. Under an Olympus SZ-CTV dissecting microscope, each E13.5 embryo was dissected from the uterus and placed in PBS in its own 35 mm tissue culture dish. Using microforceps, the viscera of the abdomen was carefully removed as not to damage the posterior abdominal wall. Once the nephric duct was identified, it was resected laterally to expose the kidney, gonad and adrenal gland. With the forceps, the kidney was gently loosened from the underlying and surrounding tissue and placed on a Nuclepore filter in an individual well of a 24-well culture plate filled with 200 μL of PBS until all embryos in the litter had been dissected. Kidneys were then fixed in 100% methanol at -20°C.

To visualize renal branching patterns, tissues were permeabilized with 0.1% Triton-X solution and non-specific binding was blocked with 10% normal donkey serum (NDS). Incubations with the polyclonal primary antibody specific for calbindin-D28k (rabbit polyclonal anti-calbindin, 1:100 dilution in PBS; Cemines, Santa Cruz Biotechnology) and secondary (Alexa 546, donkey anti-rabbit, 1:2000 dilution in PBS; Molecular Probes) antibodies were performed overnight at 4°C. Visualization was carried out under fluorescent microscopy with an Olympus BX-41 microscope (TRITC filter) and an Olympus DP-11 photographic module.

cDNA synthesis / qRT-PCR / quantification of Six2 in stock P19 cells

P19 embryonic carcinoma cells were obtained from ATCC (Manassas, VA) and were cultured in MEM Alpha Medium with 2.5% fetal bovine serum plus 7.5% calf serum.
mRNA was extracted and cDNA synthesis carried out as previously described. qRT-PCR was performed to amplify Six2. qRT-PCR reaction volumes (run in triple replicates) were previously described. A serial dilution of a Six2 plasmid was used as a standard by which the P19 cDNA was compared to establish a Six2 concentration. The thermocycle profile used was an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 15 sec (denaturation), 59°C for 30 sec (annealing), 72°C for 60 sec (extension) and 80°C for 6 sec (quantification of fluorescence). Following every extension step, fluorescence was measured using a MyIQ Single Color Real-Time PCR Detection System. Following qRT-PCR, product-specific amplification of Six2 was confirmed by electrophoresis in a 1% USB agarose gel.

Transfection of Six2 shRNA into P19 cells

Transfection of Six2 shRNA into P19 cells was accomplished using Lipofectamine 2000 Transfection Reagent (Invitrogen). One day before the transfection, $10^5$ cells were plated in 500 μl of culture medium sans antibiotics and allowed to proliferate to 90-95% confluence at the time of transfection. On the day of transfection, 0.8 μg of each of five different plasmid shRNA constructs was diluted in 50 μL of Opti-MEM and gently mixed. 2 μL of Lipofectamine was diluted in 50 μL of Opti-MEM and mixed gently. Both solutions were incubated at room temperature for 5 min. The DNA and Lipofectamine solutions were combined, mixed by pipetting and incubated for at least 20 min. The resulting 100 μL mixture was added to cell culture plate.
Quantification of Six2 in P19 cells following transfection with shRNA

First strand cDNA synthesis, from an mRNA template (mRNA concentration, 200 ng/μL), was performed as previously described. qRT-PCR was performed in triple replicates to amplify Six2 and GAPDH. Primers to amplify Six2 and GAPDH were previously described. The thermocycle profile and fluorescence quantification used was the same as when Six2 expression was measured in the stock P19 cell line. In order to analyze the knockdown efficiency of each of the shRNA constructs, the ratio of Six2 to GAPDH was calculated using the $2^{-ΔΔC(t)}$ method (Livak and Schmittgen, 2001). Specific amplification of GAPDH was confirmed with melting curve analysis.
RESULTS

Quantification of Six2 in the facial prominences

The anatomy of the facial prominences, from which mRNA was extracted, is shown in Figure 1. Of the microsatellite markers analyzed in Table 1, D17Mit76 and D17Mit56 both had only 1 recombination out of 720 mice (LOD score of 213), thereby placing the Br mutation just distal to D17Mit76 (Table 1). Thus, D17Mit76 was considered informative and used to genotype mice in subsequent analyses since it is likely to be nearest to the site of the Br mutation (Fogelgren et al., unpublished data). Genotypes were assigned based on the scoring of gels following electrophoresis. An example of a 4% Metaphor agarose gel is seen in Figure 2. For 3H1 Br/+ x Balb Br/+ or 3H1 Br/+ x Cast Br/+ crosses, a genotypic ratio of 1:2:1 (+/+ : Br/+ : Br/Br) was expected. If a gel showed what appeared to be an unusual ratio, PCR amplification of D17Mit76 was re-run and a second gel photographed to confirm genotypes.

qRT-PCR of facial prominence mRNA began with melting curve analysis to confirm the specific amplification of Six2 (Figure 3a) and GAPDH (Figure 3b). Melting curve analysis also ruled out the possibility of primer-dimers. Each curve demonstrated a single peak, confirming a single sequence had been amplified by each primer set and that the primers were satisfactory for future qRT-PCR runs.
Figure 1. Wild-type E11.5 mouse embryo during dissection of the facial prominences.
The MAX were dissected first followed by the LNP and MNP. (A,B) Anterior view showing the MNP, LNP and MAX. (C,D) Profile view showing the right MAX. (E) MNP and LNP are visible following dissection. (F) MAX following dissection.
Figure 2. Photograph of 4% Metaphor agarose gel used for genotyping.
Genotypes were scored based on the number of amplimers seen. One band at 3H1 was scored Br/Br, one band at Balb (or Cast) was scored +/-, a band at 3H1 and Balb (or Cast) was scored Br/+). Photograph taken with a Kodak Gel Logic 200 photographic module.

Figure 3. Melting curve analyses to confirm specific amplification of Six2 and GAPDH in facial prominences.
A. Melt curve for Six2. Melting point ~ 88.5°C. B. Melt curve for GAPDH. Melting point ~ 84°C.
qRT-PCR was utilized to compare Six2 expression among facial prominences (Figure 4, Table 2) and genotypes (Figure 5, Table 3) using the $2^{\Delta\Delta C(t)}$ method (Livak and Schmittgen, 2001) and GAPDH for standardization. Three sets of each prominence were run in triple replicates and C(t) values averaged. Among the wild-type prominences, the MNP showed the highest expression of Six2 and, thus, was set as the standard by which the other prominences were compared throughout the study. The expression of Six2 in wild-type MAX was less than 45% of that in the MNP while LNP displayed 29% the expression level of MNP. The heterozygous MNP displayed the highest expression level of Six2, while the MAX and LNP expression was 53% and 27% of the MNP, respectively. Among the homozygous mutant population measured for Six2 expression, the expression pattern paralleled that of the wild-type and heterozygous conditions: the MNP showed the highest expression of Six2 with the MAX expression 71% and LNP expression 58% of the wild-type.

When Six2 expression was quantified among genotypes, consistent results confirmed highest expression in each of the wild-type prominences with lower expression in the Br+/+ and lowest in the Br/Br. Among the MNP, expression of Six2 decreased by 60% in the Br+/+ and 93% in the Br/Br. LNP expression of Six2 decreased by 62% in the Br+/+ and 87% in the Br/Br. The MAX Br+/+ prominences decreased expression 50% while the Br/Br lost 89% expression of Six2.
Figure 4. Differences in Six2 expression among facial prominences in E11.5 ++, Br/+ and Br/Br embryos. (A) ++ (B) Br/+ (C) Br/Br. mRNA was extracted from facial prominences and Six2 expression measured by qRT-PCR and standardized GAPDH expression.
Table 2. Data obtained from qRT-PCR to quantify *Six2* expression in facial prominences among genotypes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Prominence</th>
<th>C(t) <em>Six2</em></th>
<th>C(t) GAPDH</th>
<th>Relative expression of <em>Six2</em></th>
<th>σ</th>
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</tr>
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</tr>
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</tr>
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<td></td>
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</tr>
<tr>
<td>MNP</td>
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<td>16.9</td>
<td>20.8</td>
<td>16.8</td>
<td>1.0</td>
</tr>
<tr>
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<td>16.8</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LNP</td>
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<td>23.5</td>
<td>17.7</td>
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</tr>
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Figure 5. Differences in Six2 expression among genotypes in E11.5 facial prominences. 
(A) MNP (B) LNP (C) MAX. mRNA was extracted from facial prominences and Six2 expression measured by qRT-PCR and standardized GAPDH expression.
Table 3. Data obtained from qRT-PCR to quantify Six2 expression in facial prominences among facial prominences.

<table>
<thead>
<tr>
<th>Prominence</th>
<th>Genotype</th>
<th>C(t) Six2</th>
<th>C(t) GAPDH</th>
<th>Relative expression of Six2</th>
<th>σ</th>
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<tr>
<td>MNP</td>
<td>+/+</td>
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<td>-17.7</td>
<td>0.13</td>
<td>0.02</td>
</tr>
<tr>
<td>LNP</td>
<td>+/+</td>
<td>21.0</td>
<td>-17.9</td>
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</tr>
<tr>
<td></td>
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<td>-18.4</td>
<td>0.11</td>
<td>0.01</td>
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</tbody>
</table>

Kidney whole mount immunohistochemical staining

Kidney dissections were carried out at E13.5 for the wild type, heterozygote and homozygous mutants as shown in Figure 6. These kidneys were immediately fixed, followed by staining for UB branches. Visualization of the UB branches was by indirect immunofluorescent microscopy using antibodies directed against the calcium-binding protein Calbindin-D28k. While absent in the renal tubules derived from the MM, metanephric UB branches contain Calbindin-D28k (McIntosh et al., 1986). Thus, Calbindin-D28k is suitable for labeling only structures derived from the UB while MM derived structures are not immunolabeled. Wild-type kidneys were expected to show the
Figure 6. Schematic of dissection protocol for removing E13.5 kidneys.

Cuts were made to isolate the abdomen from the rest of the embryo (A), the hind limbs removed (B) and somites removed and lateral resection of the nephric duct (C) allowed for visualization of the embryonic kidney. Kidneys were removed (D) with microforceps and placed in PBS (based on an illustration provided by Dr. Carl Bates, Dept. Nephrology, The Ohio State University).

greatest number of branches from the UB, while fewer branches were expected in the $Br^+/-$ and fewest in the $Br/Br$.

Figure 7 shows the branching pattern of $+/+$, $Br^+/+$ and $Br/Br$ kidneys. The greatest number of UB tips originate form the UB in the wild-type kidney (24). The number of tips decreases in the $Br^+/+$ (16) and further decreases in the $Br/Br$ (5).
Figure 7. Branching of the ureteric bud by stage E13.5.
Following staining for Calbindin-D28k, newest buds can be seen in the renal cortex (arrowheads) while older buds reside in the medulla. The ureter can also be seen (arrow).
(A) +/+ , (B) Br/+ and (C) Br/Br. Bar = 100μm.

Quantification of Six2 in stock P19 cells

In order to determine if embryonic carcinoma P19 cells expressed Six2 at a level that could be knocked-down subsequently, a plasmid serial dilution was carried out and qRT-PCR performed (Figure 8). Following qRT-PCR, the equation $y = -3.383(x) + 37.915$ was used to calculate Six2 starting quantity in the cell line.

qRT-PCR to confirm and establish Six2 starting quantity in P19 cells was performed in triple replicates and the results displayed in Table 4. The P19 cell culture system showed a Six2 concentration of $4.2E+01$ copies/μL cDNA. Melting curve analysis for Six2 demonstrated duplex targets. This was confirmed by gel electrophoresis (Figure 9).
Figure 8. Plasmid serial dilution used to quantify the expression of \textit{Six}2 in stock P19 cells. Scatterplot shows the negative correlation between starting quantity and threshold cycle. Equation for line used to calculate starting \textit{Six}2 copy number in P19 cells: $y = -3.383(x) + 37.915$.

Table 4. Data obtained from qRT-PCR to quantify \textit{Six}2 expression in P19 cells resulting from a serial dilution.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>( C(t) )</th>
<th>([\text{Six}2]) (copies/(\mu\text{L}_\text{DNA}))</th>
<th>( \text{Avg}[\text{Six}2]) (copies/(\mu\text{L}_\text{DNA}))</th>
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<tr>
<td>P19</td>
<td>32.7</td>
<td>(3.4\times10^1)</td>
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<tr>
<td></td>
<td>32.4</td>
<td>(4.2\times10^1)</td>
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<td></td>
<td>32.1</td>
<td>(5.1\times10^1)</td>
<td>(4.2\times10^1)</td>
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Figure 9. Confirmation of specific amplification of Six2 in P19 cell line.
(A) Melting curve analysis for Six2 showed two peaks indicating non-specific amplification. (B) Gel electrophoresis for PCR confirmed the second peak. Weight of Six2 amplicon, 150 bp.

Quantification of Six2 in P19 cells following transfection with shRNA

RNAi technology was applied to reduce Six2 expression in the P19 cell line. Six cultures of P19 cells were transfected (five with different shRNA constructs and one mock-transfected to provide a control) to determine if Six2 could be knocked down in a cell culture system. C(t) values obtained by qRT-PCR for Six2 and GAPDH were entered into the 2^{-ΔΔC(t)} method for normalizing gene expression (Table 4) (Livak and Schmittgen, 2001). The knock-down efficiency of each of the Six2 shRNAs are shown in Figure 10 and Table 5 for the five constructs: 37%, 12%, 15%, 53% and 32% respectively (shRNA
Figure 10. Relative expression of Six2 in P19 cells following transfection with shRNA. Five shRNA constructs (1-5) were all able to knock down Six2 expression to some degree, relative to the control, mock-transfected cells, as measured by qRT-PCR and standardized GAPDH expression. Knockdown percentages: (1) 34%, (2) 9%, (3) 12%, (4) 51% and (5) 30%.

Table 5. Data obtained from qRT-PCR to quantify Six2 expression in P19 cells following shRNA transfections.

<table>
<thead>
<tr>
<th>Identifier</th>
<th>C(t) Six2</th>
<th>C(t) GAPDH</th>
<th>Relative Six2 expression</th>
<th>σ</th>
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</table>

1-5). Specific amplification of GAPDH was confirmed by melting curve analysis (Figure 11).
Figure 11. Melting curve analysis to confirm specific amplification of *GAPDH* in P19 cells. Melting point $\sim 84^\circ$C.
DISCUSSION

Identifying phenotypes proved to be extremely difficult at E11.5, therefore, grouping of similar embryos was delayed until confirmation of genotypes could be achieved. Our lab has previously linked the Br mutation to the homeobox gene Six2, located on distal murine chromosome 17 (McBratney et al., 2003). Microsatellites were analyzed for recombination to identify a suitable primer for differentiating genotypes. D17Mit76 was determined best suited for genotyping due to its low incidence of recombination (1 recombination from 720 total backcross mice analyzed, distance between D17Mit76 and Six2 = 0.14 cM) and its PCR products yielding a significant size difference between 3H1, Balb and Cast strains that could be differentiated by agarose gel electrophoresis. Even though no recombination was observed from MS3 (720 total mice analyzed) and the PCR product size was significantly different between 3H1 and Cast strains, the size difference between 3H1 and Balb was too small to differentiate following gel electrophoresis. Thus, after electrophoresis, a single 3H1 band was scored as a Br/Br, a single Balb or Cast band was scored +/+, and bands at both 3H1 and Balb/Cast was scored Br/+.

qRT-PCR was performed on mRNA extracted from the MNP, LNP and MAX to amplify and quantify the expression of the transcription factor Six2. Transcription factors bind to promoter regions of DNA using ultra-specific binding domains and precisely regulate the transcription of relevant genes by initiating RNA polymerase. For example, homeobox genes encode transcription factors, such as Six2, whose domain (homeodomain) is responsible for binding to the promoter region and, in turn, initiating transcription of the target gene. Thus, if a genetic mutation arises in the DNA template
and compromises the expression of *Six2*, the target gene of the transcription factor may also be misexpressed.

The growth of the facial prominences is activated and regulated by specific transcription factors (Diewert and Lozanoff, 2002). Oliver et al. (1995) found expression of the transcription factor *Six2* in the developing mid-face to be localized in the neural crest-derived mesenchymal tissues of the cranium and these cells are responsible for the proximodistal growth of the frontonasal prominence (Marcucio et al., 2005). In this study, we have reported *Six2* expression is highest in the MNP of wild-type embryos and, in the developing MNP of *Br/+* embryos, *Six2* expression decreases by more than 50% while the homozygous mutant shows a 96% decrease. Fogelgren et al. (unpublished data) observed mesenchymal hypoplasia in *Br* mice, suggesting *Six2* may promote cellular proliferation in the neural crest mesenchyme. This proliferation of the neural crest mesenchyme pushes the MNP and MAX together (Senders et al., 2003). Thus, we suggest the reduction of *Six2* expression in *Br* mice results in mesenchymal hypoplasia, retarding the growth of the mid-face, leaving the merging of the paired MNP incomplete and leading to FND with the possibility of cleft palate and/or lip if the fusion of the MNP and MAX is incomplete as well.

Kidneys at E13.5 were dissected away from surrounding tissues and prepared for staining for Calbindin-D28k to visualize branching of the UB. *Six2* up-regulates morphogenetic factors such as GDNF, which are secreted from the MM to initiate UB branching (Brodbeck et al., 2004). Thus, based on the branching morphogenesis of the E13.5 *Br* kidney, we can propose the influence of *Six2* expression on renal
morphogenesis. During embryonic days E11.5 until E14.5, the UB experiences approximately 7.5 bifid branching events (Cebrián et al., 2004). The UB in the wild-type kidney demonstrated 24 UB tips, or approximately 5 branching events. The heterozygote kidney demonstrated 16 UB tips, or 4 branching events, and the Br kidney 5 tips, or approximately 2.25 branching events. While this haploinsufficient branching pattern parallels Six2 expression demonstrated in embryonic kidney (Fogelgren et al., unpublished data), our observation of one explant per genotype will need further analysis to conform our hypothesis. However, the reduced number of UB tips in the Br embryonic kidney implies a fewer number of glomeruli and thus smaller (hypoplastic) kidneys could be expected in the adult. This has been reported in Br/Br mice where Lozanoff et al. (2001) found the nephrogenic zone and associated glomerular number was greatly diminished in the homozygous mutant. Thus, an adult with a mutation in Six2 would be expected to display diminished glomerular development and numbers resulting in features of chronic renal failure including hypertension, increased filtration rate but diminished clearance and possibly polyuria. Six2 mutations in human adults might be expected to predispose a patient to renal disease since a decrease in glomerular number is associated with kidney disease (Hughson et al., 2003). Further studies should be directed to determine whether experimental animals display chronic renal failure.

shRNA transfection begins by introducing a vector containing the shRNA sequence into a cell culture system. Lipofectamine 2000 (Invitrogen) was used as a transfection reagent to alter the plasma membrane such that the shRNA could enter the cytoplasm. Once inside the cell, shRNA is cleaved by dicer RNase into 20-25 nucleotide long small
interfering RNA (siRNA). Each fragment of siRNA can bind with RNA induced silencing complex (RISC) proteins, where the siRNA fragment becomes the guide strand to which complementary, endogenous mRNA can base pair. The endogenous mRNA is then degraded via argonaute endonuclease, preventing the translation of the mRNA and, thus, silencing the target gene, Six2. Therefore, a siRNA approach could be expected to down-regulate Six2 in vitro.

When P19 embryonic carcinoma cells are isolated into culture, they grow indefinitely in a monolayer as an undifferentiated cell line (Martin and Evans, 1974, 1975; Martin, 1975). As shown in Figure 7, P19 cells indeed express the transcription factor Six2. These attributes make P19 cells a suitable candidate for shRNA mediated knock-down of Six2. However, the presence of a non-specific product may have improperly augmented the fluorescence in the qRT-PCR, and, in turn, the calculated Six2 concentration. We hypothesized that a preliminary reduction of gene expression in the P19 cell culture system by shRNA by at least 50% should be effective enough to use on whole organ cultures. Of the five shRNA constructs we tested, one construct did, in fact, reduce Six2 expression by about 50%.

Future experiments will involve the transfection of shRNA into facial prominence cell culture systems. We have already begun work on establishing cell culture systems for MNP, LNP and MAX to perform knock-down experiments. Using the most effective shRNA construct (and combinations of constructs), we will attempt to transfect the facial prominences and quantify the reduction in Six2 expression in vitro. When we can confirm knock-down in all of these cell culture systems, we will attempt whole organ
knock-downs of the kidneys and face. We have already established a successful culture system for E12.5 kidneys.

Self et al. (2006) has shown the knockout effects Six2 in mice in vivo. These knockout mice demonstrated the renal hypoplasia phenotype associated with the Br mutation, however, no observation of FND was reported. Furthermore, Six2+/− mice did not display any difference in phenotype from wild type mice and Six2 expression in the craniofacial tissues of knockout mice was not reported. In fact, little is known of the mechanisms by which Six2 is active in the craniofacial tissues. If we can incorporate our RNAi technology into an in vivo situation, we could quantify and compare Six2 expression in the developing mid-face and kidney of Six2 knock-down and Br/Br and Br/+ embryos to better understand the relationship between Six2 expression, frontonasal dysplasia and renal hypoplasia.
REFERENCES


