Essential Techniques Required for Optimal Observation of Forkhead Transcription Factors FOXO1 and FOXO3 and Their Role in Pituitary Gland Development and Function.

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Introduction

Transcription factors are proteins or compounds that are capable of inhibiting or enhancing the rate at which genes are transcribed. Forkhead box transcription factors possess a DNA binding domain that binds the promoter and is a double winged helix, which acts as the enhancing region of specific genes. FOXO1 and FOXO3 are closely related forkhead box transcription factors (**Figure 1**) that aid in the role of somatotroph differentiation. Somatotropes are the cells that produce growth hormone and are located in the anterior region of the pituitary gland. Genetically modified mouse models are used for observing the role of these forkhead transcription factors in pituitary gland development and function. In this study, various techniques are performed to establish optimal results for utilizing mouse models for research. Foxo1 is expressed in the pituitary gland, heart, and placenta. Results from Stallings et al demonstrate that the timing of FOXO1 activation affects its role in pituitary gland organogenesis and somatotrope differentiation [5]. Cre-lox mediated technology is used to promote tissue specific-deletion of *Foxo1* in the pituitary gland. Expression is then analyzed and measured at various embryonic stages using Immunohistochemistry (IHC) under fluorescent microscopy. *Flox*(f/f) is an indication that the *Foxo1* gene was "floxed" by inserting loxP sites (**Figure 2**). The use of cre deletes the floxed *Foxo1* gene. Mice are mated based on their genotype, which is determined through genotyping analysis performed via PCR and gel electrophoresis. Each mouse has genotype that is either homologous or heterozygous for *Foxo1*, *Foxo3*, and *Fog1* with or without the presence of cre. For example, a mouse model could have the following genotype: $Foxo1^{+/f}$, $Foxo3^{f/f}$ $Foxg1^{+cre}$. *Foxg1*-cre stimulates pituitary specific deletion, because *Foxg1* is expressed in the pituitary gland causing cre to be present in the pituitary gland. This method prevents the demise of mice, if Foxo1 deletion were to occur everywhere, this cause's early embryonic lethality.

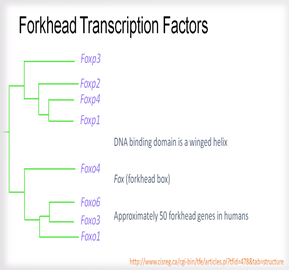


Figure 1. Forkhead box tree--Foxo3 and Foxo1 appear to be closely related transcription factors. There are roughly 50 forkhead transcription factors in humans, and about 44 in mice.

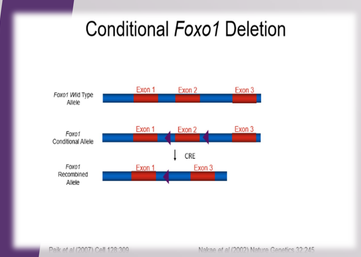


Figure 2. Displays the new recombined allele with the presence of cre and the use of inserting lox-p sites. When cre is introduced the site specific region between the lox-p sites the section is then deleted. The lox-p sites then ligate together creating the new recombined allele.

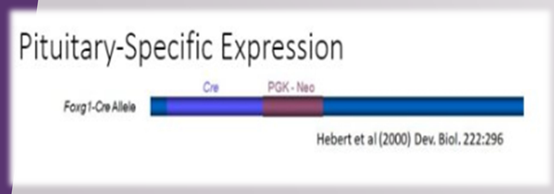
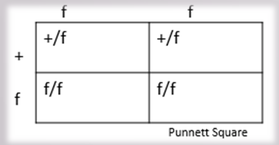
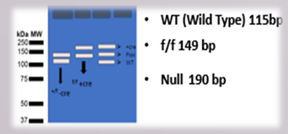


Figure 3.Foxg1-cre stimulates pituitary specific deletion

Methodology

- WT/dKO mice
- Biopsy of tail or ear
- DNA isolation
- Genotyping
- PCR/ Gel Electrophoresis
- Pituitary gland--paraffin wax preservation
- Tissue sectioning with microtome
- Immunohistochemistry
- Primary antibodies
- Secondary antibodies
- SA-HRP
- Alexa Fluor
- Fluorescent Microscopy

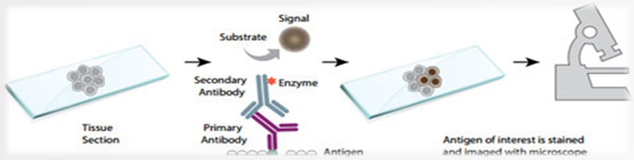




Genotyping using PCR and Electrophoresis Polymerase chain reaction is a technique that takes a small sample of DNA and amplifies it. This is one of the methods used in this research to determine if mouse models contain the wild type allele or have been successfully knocked out also referred to as DKO mice.

Immunohistochemistry (IHC) is an important application of monoclonal as well as polyclonal antibodies to determine the tissue distribution of an antigen of interest in health and disease. [6] Which used to amplify the expression of the targeted gene protein and confirm the success of knockout mice gene deletion.

Results



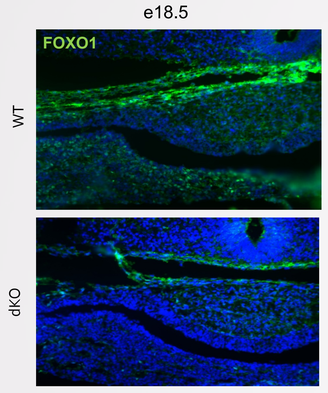
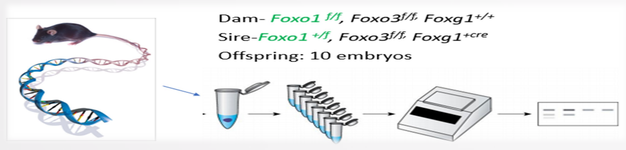


Figure 4. Immunohistochemistry preformed on e18.5 coronal section of pituitary tissue samples using Tyramide SuperBoost kit with Alexa Fluor Tyramides. Fluorescent microscopy with bulbs FITC—tinted green, and DAPI—tinted blue; were used to capture the expression on the pituitary sample. This procedure was done to visualize if successful deletion of *Foxo1* occurred; based on embryos that were genotyped using PCR and electrophoresis. Nuclei were stained with DAPI (blue); alexa fluor tyramide (green) was used to tag the primary antibody *Foxo1*. Which is seen in the wild type (WT) sample. dKO represents double knockout—deletion of Foxo1. In the dKO pituitary tissue sample it's evident there is no presence of green positive cells in the posterior and anterior lobe indicating *Foxo1* deletion had indeed occurred. Slides were incubated for 5 minutes using the Tyramide solution.



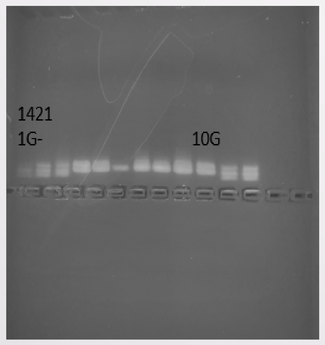


Figure 5.Two strains of mice have been mated with the genotypes as listed: Dam: Foxo1f/f , Foxo3f/f, Foxg1+/+ Sire: Foxo1+/f, Foxo3f/f , Foxg1+cre . Ten embryos were then collected from the mother and a biopsy was preformed collecting a portion of each tail. From here the DNA was isolated using lysogenic and neutralizing buffers and then prepared for PCR After collecting PCR samples, they were then loaded onto a 2% agarose gel and ran through electrophoresis, where the migration of distinct bands was visualized. These bands will determine the genotype for each embryo. The results obtained indicated that 60% of the offspring were Foxo1f/f, Foxo3f/f, Foxg1+cre, 30% were Foxo1+/f, Foxo3f/f Foxg1+cre, and 10% Foxo1f/f, Foxo3f/f Foxg1+/+.

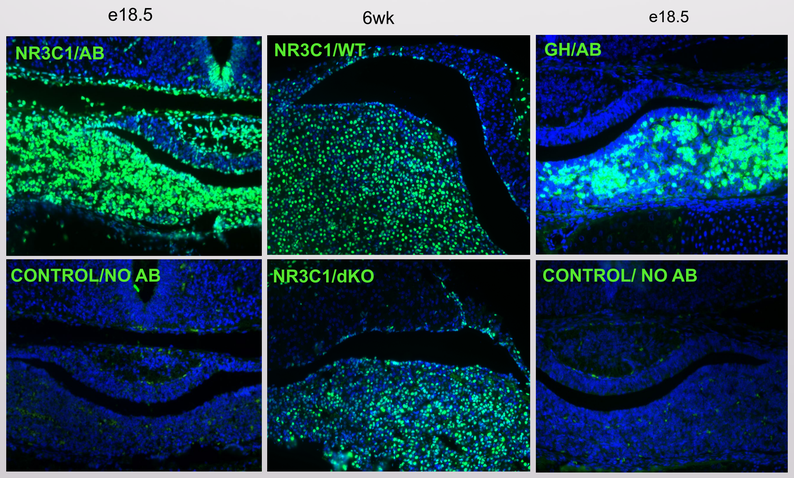


Figure 6. Immunohistochemical analysis with the use of gluccocorticoid receptor and growth hormone. **Section one:** IHC performed on two slides at a five-minute Tyramide fluoresence incubation period—each with two e18.5 coronal pituitary tissue sections. Slide one was treated with primary antibody NR3C1 on one of the e18.5 pituitary tissue sections; with the second section being a control sample without a primary antibody. NR3C1 is the glucocorticoid receptor. Both samples were incubated for five minutes with the Tyramide solution (Green—FITC) and then stained with DAPI (blue). The control without NR3C1 as the primary antibody indicates that NR3C1 attached properly to positive cells rather than falsely binding to hemaglobin cells and/or tissue. The procedure was then repeated **(section 2)** on 6-week pituitary samples also with a five-minute incubation period with the Tyrramide fluorescence; one wild type(WT) and one double knockout (dKO). Resulting in no obvious difference regarding the presence of *Foxo1* and without *Foxo1*. This indiciates that presence of *Foxo1/3* has no effect on NR3C1's expression. **Section three:** Growth hormone signaling– somatotropes secrete growth hormone (GH), which regulates growth and metabolism [5]. e18.5 pituitary tissue section treated with growth hormone as the primary antibody and a control pituitary section with no primary antibody treatment. These sections were incubated for 10 minutes with the Tyramide solution (green—FITC).

Conclusion

These techniques have proved to be extremely useful in various ways like: identifying somatotrope cells at different embryonic ages, determining the genotype of offspring mated with cre mice, and visualizing expression of forkhead transcription factors FOXO1 and FOXO3 and whether or not they have been successfully knocked out. Regional genetic deletions of Foxo1/3 in the pituitary gland results in a decrease of hypermutations. Expression of *Foxo1* can be seen as early as embryonic day e10.5. It's essential to observe this expression amongst various embryonic stages for the purpose of understanding *Foxo1*'s role in pituitary gland development and function. It's also crucial to observe the growth hormone signaling of somatotrope differentiation in *Foxo1* early embryonic deletion . Glucocorticoid receptor NR3C1 and GH studies indicated that there's no broadly apparent difference in expression from mice lacking *Foxo1* and *Foxo3.*

Future Directions

Exploration of other useful techniques such as creating an assay using Real Time Polymerase Chain Reaction (RT-pcr); which is an important tool for analysis of RNA. This tool enables measurement of mRNA levels by reverse transcription. [2]

Other future directions include: identifying different protein coding genes, adjusting incubation times with the Alexa Fluor, and analyzing growth hormone signaling of expression at different embryonic stages.

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