**Measurement of the p38 signaling pathway in the Pro253Arg mutation of FGFR2 in regulation of bone development in Apert Syndrome**

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**I. Introduction**

Birth defects are a severe problem that affect 1 in every 33 newborns in the U.S. and account for 20% of all infant deaths, making it the leading cause of infant mortality in the United States. They can vary from being mild to severe structural changes that occur in any part of the body and affect how the body looks, functions, or both. Depending on the severity of the birth defect, it can not only lead to infant mortality, but it can also lead to disability and a significant reduction of a child’s lifespan (Center for Disease Control and Prevention, 2016).

Craniofacial defects account for almost one-third of all birth defects. Of these, Craniosynostosis (CS) is one of the most common craniofacial anomalies that occurs in approximately 1 in 2,500 live births. CS is characterized by the premature fusion of one or more cranial sutures in the skull, which can result in abnormal head shapes due to restricted skull growth. It can be classified as primary CS, resulting from intrinsic genetic causes, or secondary CS, resulting from disorders affecting the developing sutures. Primary CS can further be classified as nonsyndromic or syndromic. Nonsyndromic CS has no additional abnormalities or developmental delays and occurs in approximately 75% of patients with CS. On the contrary, syndromic CS is associated with other abnormalities and/or developmental delays and occurs in the remaining 25-30% of patients (Lattanzi, Barba, Di Pietro, & Boyadjiev, 2017).



**Figure 1** Craniofacial features of individuals with craniosynostosis.

(Lattanzi, Barba, Di Pietro, & Boyadjiev, 2017)

Bones are formed through two processes known as intramembranous ossification, which generates bone tissue, or endochondral ossification, which generates both cartilage and bone tissue. Abnormalities in these processes are what lead to diseases such as CS. Apert syndrome (AS), one of the most severe forms of syndromic CS, accounts for around 4.5% of all CS cases (Yin, et al., 2008). Its symptoms can include abnormalities of the brain, skin, and visceral organs, depressed nasal bridge, oral deformities, syndactyly of the digits, mental retardation, and respiratory complications (Chen, Li, Li, Engel, & Deng, 2003). The majority of AS is caused by mutations in adjacent amino acids- a Ser252Trp mutation or Pro253Arg mutation in the fibroblast growth factor receptor 2 (FGFR2). (Chen, et al., 2014). A typical FGFR consists of an extracellular ligand-binding domain, a transmembrane region, and a divided intracellular domain. When a fibroblast growth receptor (FGF) binds and activates the FGFR, target proteins are brought to the intracellular domain and phosphorylated, leading to the activation of the several signaling pathways (Su, Jin, & Chen, 2014). The two mutations mentioned above both increase the affinity and alter the specificity of FGF ligand binding (Chen, et al., 2014).

FGFs and their receptors (FGFRs) are known to play an important role in endochondral bone formation. FGFR2, specifically, is responsible for activating the mitogen-activation protein kinase (MAPK) pathway, which includes the Erk1/2 and p38 signaling pathways. Since endochondral bone formation is dependent on the MAPK pathway, mutations in FGFs and FGFRs cause abnormalities in bone formation, resulting in disorders such as AS (Chen, et al., 2014). Although recent studies suggest that the MAPK signaling pathway plays a role in bone development, the specific signaling mechanisms that cause the positive effects of the Ser252Trp and Pro253Arg mutation in FGFR2 remain unclear.

Chen, et al. (2014) investigated the role of the Erk1/2 and p38 signaling pathways in bone development using bone mesenchymal stem cells (BMSCs) of mouse models with the FGFR2 Ser252Trp mutation mimicking AS. It was speculated that these pathways play a role in the resulting phenotypes caused by the mutation. Their experiments found that both the Erk1/2 pathway and p38 pathway were activated. In addition, they were also able to conclude that the Erk1/2 pathway promotes the late stage of chondrogenic, or cartilage, differentiation of BMSCs and that the p38 pathways influences the entire endochondral ossification process, making the latter signaling pathway more important in pathogenesis of AS.

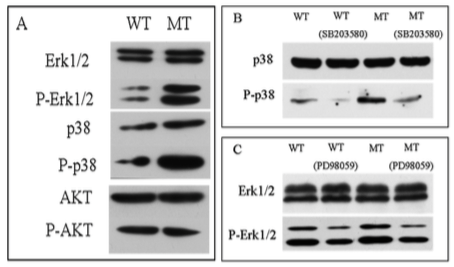
Similarly, Yin, et al. (2008) investigated the involvement of the Erk1/2 signaling pathway in the pathogenesis of bone abnormalities in mouse models mimicking AS, except with the Pro253Arg FGFR2 mutation. Using the *in vivo* mouse models, they found that inhibiting the Erk1/2 pathway prevented the premature closure of coronal sutures in the mice and partially recovered the retarded endochondral ossification. These findings suggest that the Erk1/2 pathway is involved in the abnormal bone development induced by the Pro253Arg FGFR2 mutation.

As described above, the involvement of the Erk1/2 signaling pathways has been investigated in the Ser252Trp and Pro253Arg mutation of FGFR2. The role of the p38 signaling pathway has also been explored, but only in the FGFR2 Ser252Trp mutation. The purpose of the experiment described in this proposal is to determine if the p38 signaling pathway is involved in the regulation of bone development in the Pro253Arg mutation of FGFR2.

**II. Experiment**

The aim of this experiment is to determine if the p38 signaling pathway is involved in the pathogenesis of AS, specifically in the regulation of bone development with the Pro253Arg mutation of FGFR2. In order to do this, BMSC’s from mouse models with the Pro253Arg will be extracted and used in a western blot analysis to measure the levels of activation and concentration of p38. This experiment is modeled after a western blot technique used by Chen, et al. (2014).

A. Model Experiment



**Figure 2** Western Blot Results (Chen, et al., 2014)

Chen., et al. (2014) used cultured BMSC’s from mice with the Ser252Trp mutation. The protein concentrations of p38 and phospho-p38 were determined in these samples and then separated by size through gel electrophoresis. The separated proteins were then transferred onto a membrane and marking using antibodies specific for p38 and phospho-p38. The results showed increased protein levels of phospho-p38 compared to p38 in mutant cells, signifying that the p38 signaling pathway was indeed activated due to the Ser252Trp mutation.

B. Cells obtained from mouse models

Mice are commonly used organisms in preclinical studies because with their genome being 99% similar to the human genome, they provide a means to study disease mechanisms in complex organisms (Vandamme, 2014). Their small size also makes them a cost efficient model because they allow for large scale and high quantity studies. Using a mouse model, for this experiment in particular, allows us to reproduce organisms with the desired traits (FGFR2 Pro253Arg mutation) and provides a method to understanding the pathways underlying AS.

In order to conduct this experiment, mouse models with with the Pro253Arg mutation will be used, specifically six to eight week old mice. Bone marrow from the tibiae and femora will be flushed out and cultured for 14 days to use in the western blot analysis.

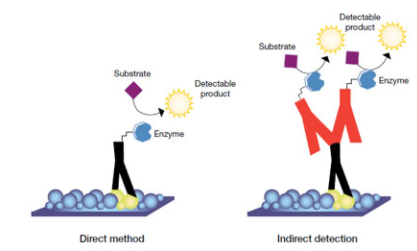
C. Western Blot Analysis

Western blots are used to separate and specifically identify protein from a mixture of various proteins that are also extracted from particular cells. After culturing, the BMSCs will be extracted and the protein concentrations in the cell will be determined using a protein assay kit. Equal amount of the proteins will be placed on a gel for gel electrophoresis. In this process, the molecules are pushed by an electric field that separates them based on size- smaller molecules will travel further down the gel and larger molecules will travel less further.

The size separated proteins on the gel will then be transferred and sandwiched on a polyvinylidene fluoride (PDVF) membrane, a process known as electrophoretic transfer because the negatively charged proteins are transferred onto a positively charged membrane. The membrane will be blocked to prevent any nonspecific binding of antibodies to the surface. The target proteins (p38 and phospho-p38) are then marked with their appropriate primary antibodies followed by secondary antibodies in order to visualize the phosphorylated protein level in comparison to the total protein level of p38 (Mahmood & Yang, 2012).

**III. Discussion**

The expected, and ideal, outcome of this experiment is that the western blot will show increased protein levels of phospho-p38 compared to p38 in the mutant BMSCs. This result would suggest that the p38 signaling pathway is activated with the Pro253Arg mutation due to the higher levels of the phosphorylated p38 protein, and would mean that this signaling pathway is indeed somehow involved in the regulation of bone development in AS. On the other hand, the results of the western blot could also show that there is no difference in the protein levels of phospho-p38 and p38, suggesting that the p38 signaling pathway is not involved in the regulation of bone development in the Pro253Arg mutation of FGFR2.



**Figure 3** Direct method (left) and indirect method (right) of detection

(ThermoFisher, 2016)

In the experiment described above, an indirect detection method for the western blot will be used instead of a direct method. An indirect method uses an unlabeled primary antibody followed by a secondary antibody to help detect it. There are more advantages to using an indirect method over the direct method of detection which include a large selection of secondary antibodies and signal amplification by the secondary antibody. Using a secondary antibody also does not inhibit target binding of the primary antibody and allows for the option of multiple detection methods (ThermoFisher, 2016). Although there are some disadvantages to this western blot technique, including a longer process with additional steps involved for staining, the benefits of the indirect detection method outweigh its disadvantages and even the benefits of the direct method, which is why it is more often used.

After completing this experiment and determining whether the p38 signaling pathway is involved in bone development, potential next steps could include figuring out what exactly in bone development this pathway affects, such as chondrogenic differentiation or endochondral ossification. Knowing whether the p38 signaling pathway is activated and involved in the Pro253Arg mutation of FGFR2 could also pave the path for discovering new treatment methods involving p38 modulators in the treatment of apert syndrome and craniosynostosis.

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