AAO Foundation Final Report Form

Type of Award: Research Aid Award

Name(s) of Principal Investigator(s): Dr. Spencer Crouch (resident) and Dr. Nan Hatch (faculty)

Title of Project: Investigating Local vs. Systemic Effects of Tissue nonspecific Alkaline Phosphatase (TNAP) on Bone Formation and Mineralization

Period of AAOF Support 07-01-18 to 06-30-19

Amount of Funding: $5,000

Summary/Abstract: See attached pages

Abstract

Background: Hypophosphatasia (HPP) is a rare genetic disorder characterized by diminished mineralization of bone and teeth. HPP is caused by loss-of-function mutations in the gene (*Alpl*) that encodes tissue non-specific alkaline phosphatase (TNAP). TNAP is an essential enzyme for mineralization that is expressed in fully differentiated bone and tooth forming cells. Mice that lack TNAP (*Alpl*$^{-/-}$ mice) serve as an important research model of HPP, exhibiting many characteristics seen in HPP patients including long bone and tooth defects, seizures, cranial base defects and craniosynostosis (the premature fusion of cranial bones). Recent data from our laboratory indicates that TNAP may be essential at the local cellular level to promote normal osteoblast progenitor cell renewal and differentiation.

Objective: The primary purpose of this study is to determine if TNAP is essential at the local, cellular level for bone formation and mineralization.

Methods: Bone marrow stromal cells (BMSCs) from long bones were isolated from TNAP deficient (*Alpl*$^{-/-}$) and wild-type (*Alpl*$^{+/+}$) mice, then mixed with a three-dimensional collagen carrier and implanted subcutaneously into host immunocompromised mice. After eight weeks *in vivo*, bone ossicles were removed and assessed for bone formation and mineralization. Radiographs were taken to calculate densitometry values. Nano CT scans of each ossicle were completed to qualitatively evaluate isosurface renderings and quantitatively evaluate parameters of trabecular and cortical bone formation and mineralization. Histological evaluation of ossicles was performed by H&E, trichome, and alkaline phosphatase enzyme stains.

Results: TNAP deficient mice utilized for BMSC isolation were significantly smaller than their wild-type littermates and produced fewer BMSCs in their long bones due to a combination of diminished size and tissue obstruction in the mid-diaphyseal region of mutant bones. Densitometry values of radiographs were significantly less for the TNAP deficient than wild-type ossicles, indicating less mineralized tissue formed when compared to the wild-type ossicles. Nano CT isosurface renderings showed that wild-type ossicles formed a cortical shell with extensive trabecular bone formation while TNAP deficient ossicles formed a cortical shell with little to no trabecular bone formation. Nano CT analyses also showed that the TNAP deficient BMSCs produced significantly diminished quantity and quality of cortical and trabecular bone when compared to wild-type BMSCs. Histologic evaluation suggests that BMSCs from both genotypes formed bone, with little to no trabecular bone formed in TNAP deficient ossicles. In addition, the marrow space of TNAP deficient ossicles showed less red marrow (hematopoietic cells) and more yellow marrow (adipocytes), when compared to wild-type ossicles. Alkaline phosphatase staining revealed diminished AP enzyme activity in TNAP ossicles.

Conclusions: TNAP was found to be needed at the local, cellular level for proper bone mineralization to occur. Initial bone formation can occur in the absence of localized TNAP, however, local TNAP is crucial for formation of trabecular bone and complete bone mineralization, even when normal levels of systemic TNAP and P_i are present.

Response to the following questions:

1. Were the original, specific aims of the proposal realized?

Yes, the original, specific aims of the proposal were realized. Due to a limited sample size of TNAP KO cells however, we did choose to eliminate the *in vitro* experiments from this Master's thesis project. *In vitro* experiments will be conducted prior to final publication.

Rationale

Infantile hypophosphatasia is a rare debilitating condition that not only affects the quality of life for the individual but may also lead to death. Results from this study will distinguish the local versus systemic effects that TNAP and P_i truly have on bone growth and development. Such results will offer pertinent information about the etiology and pathogenesis of hypophosphatasia and craniosynostosis, and the mechanistic role that TNAP has in these conditions. Establishment of TNAP as a local mediator of bone formation and mineralization may also lead to future studies in which local delivery of TNAP can be used as a bone anabolic factor to control orthodontic tooth movement.

Specific Aim

Determine if Tissue Nonspecific Alkaline Phosphatase (TNAP) enzyme is essential at a local cellular level for bone formation and mineralization using an *in vivo* approach.

Hypothesis

Subcutaneous implantation of TNAP-deficient BMSCs via a collagen carrier will significantly diminish bone formation and bone mineralization *in vivo* when implanted into a host mouse with normal systemic TNAP and P_i levels.

2. Were the results published?
    a. If so, cite reference/s for publication/s including titles, dates, author or co-authors, journal, issue and page numbers
    b. Was AAOF support acknowledged?
    c. If not, are there plans to publish? If not, why not?

The results of this study have yet to be published in a peer-reviewed journal. It has fulfilled the requirements of a University of Michigan Master's Degree project and will be included in the University of Michigan Deep Blue library. Our laboratory is in the process of conducting originally proposed *in vitro* experiments to supplement and verify these *in vivo* results. Once finished, cumulative results will be published and AAOF support will certainly be acknowledged.

3. Have the results of this proposal been presented?
    a. If so, list titles, author or co-authors of these presentation/s, year and locations
    b. Was AAOF support acknowledged?
    c. If not, are there plans to do so? If not, why not?

The results of this study were presented at the 2019 Charley Schultz Resident Scholar Award research competition at the AAO Annual Session in Los Angeles, California and was awarded first place in the biological sciences category. Below is a copy of the poster submission. AAOF support was acknowledged.

The results of this study were also presented for completion of my Master's of Science thesis project at the University of Michigan.

Investigating Local vs. Systemic Effects of Tissue Nonspecific Alkaline Phosphatase (TNAP) on Bone Formation and Mineralization

Spencer B. Crouch, DDS - University of Michigan School of Dentistry

INTRODUCTION

Background: Hypophosphatasia (HPP) is a rare genetic disorder characterized by diminished mineralization of bone and teeth. HPP is caused by loss-of-function mutations in the gene (Alpl) that encodes tissue non-specific alkaline phosphatase (TNAP). TNAP is an essential enzyme for mineralization when it is expressed in fully differentiated bone and tooth forming cells. Mice that lack TNAP (Alpl-/- mice) serve as an important research model of HPP, and exhibit many characteristics seen in HPP patients including long bone and tooth defects, seizures, cranial base defects and craniosynostosis (the premature fusion of cranial bones). Recent data from our laboratory indicates that TNAP has an essential function in bone progenitor cells at the local cellular level to promote osteoblast progenitor cell proliferation and differentiation.

Objective: The primary purpose of this study is to determine if TNAP is essential at the local, cellular level for bone formation and mineralization

Significance:

- Distinguish local vs. systemic effects that TNAP has on bone formation and mineralization
- Contribute pertinent information about the pathogenesis of HPP and craniosynostosis : role of TNAP
- Establishment of TNAP as a local mediator of bone formation/mineralization may lead to future studies in which local delivery of TNAP can be used as a bone anabolic factor to control orthodontic tooth movement and/or diminish relapse (increase bone growth + mineralization)

Hypothesis: Subcutaneous implantation of TNAP deficient BMSCs via a collagen carrier will significantly diminish bone formation and bone mineralization *in vivo* when implanted into a host mouse with normal TNAP and inorganic phosphate levels.

ACKNOWLEDGEMENTS

I would like to thank the following groups and organizations for their generous grant support of this project: The American Association of Orthodontists Foundation (AAOF), Delta Dental Foundation, University of Michigan Rackham Graduate School, and University of Michigan School of Dentistry.

METHODS & RESULTS

Approach:

1) Bone marrow stromal cells (BMSCs) were isolated from TNAP deficient (TNAP KO) and wild type (WT) mice on a 129/SVJ background– TNAP KO has severe phenotype on this background (similar to infantile HPP)
2) BMSCs amplified to passage 3 were mixed with a three-dimensional collagen carrier and implanted subcutaneously into immunocompromised mice with normal systemic levels of TNAP and inorganic phosphate
3) Implants remained in vivo for 8 weeks to allow for bone formation and mineralization
4) Removed and analyzed: radiographs, nano CT, AP enzyme stain, Trichrome and H&E

Fig. 1 Height and Weight of Donor Mice.

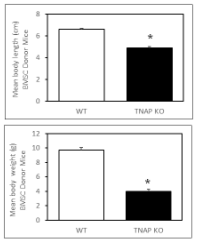

Fig. 2. Bone Marrow Stroma Cell Isolation.

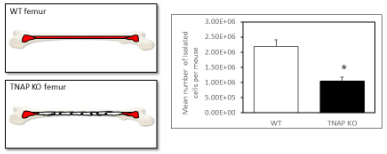

Fig. 3. Surgical Implantation

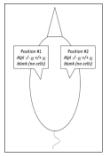

Fig. 4. Radiographs of WT and TNAP KO Ossicles.

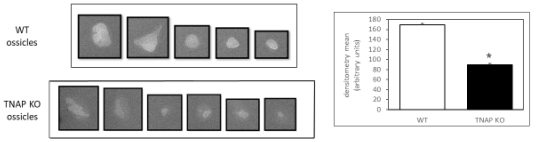

Fig. 5. Ossicle Histology.

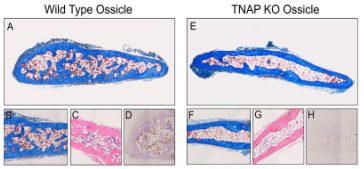

Fig. 6. WT and TNAP KO Ossicle Nano CT Isosurface Images

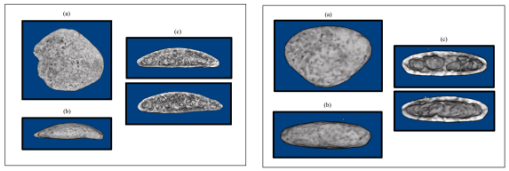

Table 1: Cortical bone analysis in WT (n = 5) and TNAP KO (n = 6) ossicles.

Parameter	Wild Type (WT) Ossicles	TNAP Knockout (KO) Ossicles	p-Value
Bone Volume (mm^3)	1.48 ± 0.34	0.51 ± 0.14	0.02*
Bone Volume Fraction	0.96 ± 0.04	0.92 ± 0.03	0.44
Bone Mineral Content (mg)	3.90 ± 0.88	0.70 ± 0.14	<0.01*
Bone Mineral Density (mg/cc)	2879 ± 512	1465.62 ± 198	0.03*
Tissue Mineral Content (mg)	3.89 ± 0.88	0.69 ± 0.14	<0.01*
Tissue Mineral Density (mg/cc)	2937 ± 482	1551 ± 178	0.02*
Average Cortical Bone Thickness (mm)	0.100 ± 0.006	0.066 ± 0.007	0.01*
Average Cortical Bone Thickness Normalized to Whole Ossicle ROI	0.040 ± 0.010	0.097 ± 0.031	0.14
* Indicates statistical significance (P <0.05) between genotypes			

Table 2: Trabecular bone analysis in WT (n = 5) and TNAP KO (n = 6) ossicles.

Parameter	Wild Type (WT) Ossicles	TNAP Knockout (KO) Ossicles	p-Value
Bone Volume (mm^3)	2.54 ± 0.57	0.58 ± 0.18	0.01*
Bone Volume Fraction	0.80 ± 0.02	0.55 ± 0.06	0.02*
Bone Mineral Content (mg)	5.68 ± 0.00	0.81 ± 0.19	<0.01*
Bone Mineral Density (mg/cc)	1983 ± 368	901 ± 184	0.02*
Tissue Mineral Content (mg)	5.55 ± 1.37	0.77 ± 0.18	<0.01*
Tissue Mineral Density (mg/cc)	2306 ± 331	1487 ± 191	0.05
* Indicates statistical significance (P <0.05) between genotypes			

DISCUSSION

This study established the local cellular role that TNAP has on bone formation and mineralization.

1. **BMSC Isolation:** TNAP KO long bones yielded fewer BMSC's likely due to both diminished bone size and bone marrow ablation.
2. **Radiographic:** TNAP KO ossicles were less radiodense indicating less mineralized bone formation. This initial data suggested that BMSCs from TNAP KO mice have less osteogenic potential than BMSCs from WT mice or simply form bone that is less mineralized.
3. **Nano CT**: TNAP KO ossicles formed a cortical shell with very little trabecular bone formation whereas WT ossicles formed a cortical shell with extensive interior trabeculation. An absence of local TNAP led to diminished bone mineralization in both cortical and trabecular bones. Cortical but not trabecular bone formation occurred when local TNAP was absent.
4. **AP Enzyme Stain:** TNAP KO ossicle did not stain for AP enzyme activity – this data, paired with radiograph and nano CT results, suggests that either other enzymes compensate for a deficiency of TNAP to promote the cortical but not trabecular bone formation process in the TNAP KO ossicles, and/or that systemic TNAP aids in the formation of cortical bone but is unable to promote trabecular bone formation.
5. **Trichrome and H&E Stain:** The marrow space of TNAP KO ossicles showed a diminished amount of red blood cells and a higher percentage of fat cells when compared to the WT ossicles – this data could indicate that TNAP promotes hematopoietic over adipocytic mesenchymal cell differentiation when expressed locally at the cellular level.

CONCLUSIONS

- TNAP was found to be needed at the local, cellular level for proper bone mineralization to occur – diminished overall mineralization of ossicles that were grown *in vivo* in an environment that had normal systemic levels of TNAP and inorganic phosphate
- Trabecular bone formation and mineralization were severely diminished by the absence of localized TNAP
- Cortical bone mineralization was severely diminished by the absence of localized TNAP, but formation was not

4. To what extent have you used, or how do you intend to use, AAOF funding to further your career?

   The Research Aid Award allowed us to study and determine the systemic vs. local role that Tissue Nonspecific Alkaline Phosphatase (TNAP) has on bone formation and mineralization. Support from the AAOF allowed us to perform integral aspects of this study and completion of this study would not have been possible without the AAOF's generous support. This support and funding provided me with an incredible research experience while at the University of Michigan that further enhanced my orthodontic education. This Research Aid Award provided me with first-hand experience in appreciating everything the AAOF truly does for residents beginning their orthodontic careers. I am extremely grateful for the generous support I have received from the AAOF thus far in my short orthodontic career.