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TITLE: Enhanced Bone Formation in Segmental Defects with BMP2 in a Biologically Relevant Molecular Context

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14. ABSTRACT
The goal of this proposal is to accelerate BMP2-induced bone formation by presenting the growth factor in a more biologically relevant context. This is based on our observation that the pentameric matricellular protein COMP binds up to five BMP2 molecules, enhancing the osteogenic response. The original proposal involved obtaining commercially available radioactively (^{125}I) BMP2 to establish release kinetics of BMP2:COMP for optimal bone formation. However, this product is discontinued. We took an alternative approach to optimize the formulation of BMP2:COMP to maximally induce bone. This involved systematically testing different formulations of BMP2:COMP, both in-vitro and in cell-based experiments, to obtain the highest osteogenic response. We originally hypothesized the strongest osteogenic response is with BMP-saturated COMP, in other-words, with 1 BMP attached to each of the 5 C-termini of the COMP protein. Surprisingly, more robust osteogenesis occurred with less BMP2 per COMP. Our revised hypothesis is that optimal osteogenesis occurs with at least 1 'arm' of COMP free to bind other molecules. Manganese did not further enhance in-vitro osteogenesis, despite earlier published observations that manganese increases the binding of BMP2 to COMP. The next steps are to validate these observations using BMP2:COMP on HA/PLG scaffolds in-vitro, then test the scaffolds in-vivo using the rat segmental bone defect model.

15. SUBJECT TERMS
Osteogenesis, BMP2, Growth Factor Presentation, Segmental Bone Defect

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Introduction

Segmental bone defects occur frequently from high impact trauma, such as gun shots. These do not heal on their own once a 'critical size' segment of bone is missing. One strategy to induce healing is to use bone-inducing proteins (BMPs), that are soaked into soft collagen sponges and placed into the area of missing bone. While this approach works, massive doses of the BMPs are required, sometimes 100,000 times the dose of BMPs that occur naturally. These high doses have side-effects that can be very severe. In thinking about why such high doses are required, we hypothesized that it is because the BMPs are not provided in a biological context that is used by the body. A BMP-binding protein (COMP) was recently identified that is a natural component of tissues that are involved in bone formation. There is good evidence that BMPs bound to COMP protein have greater bone-forming activity than unbound BMPs. Our goal in this proposal is to use this BMP-binding protein as a natural molecular "context" for the BMPs. We will test whether providing the BMPs bound to COMP will reduce the amount of BMP that is required for healing of critical size segmental bone defects. This will ultimately be done in a rat model (Aim 2). Before animal model experiments can be performed, in Aim 1 we will first establish a formulation for the BMP:COMP complexes that optimally induces bone-forming activity.

Keywords

Bone formation, Cartilage Oligomeric Matrix Protein COMP, Hydroxyapatite HA, Poly(lactide-co-glycolide) PLG, rat femoral critical size bone defect, osteogenesis, Bone Morphogenetic Protein-2 BMP2.

Accomplishments

Major Goals of the Project:

The statement of work lists the following **major goals:**

Specific Aim 1: Determine the contribution of ceramic content on the release kinetics and bioactivity of BMP2/COMP from composite scaffolds.

Major task 1: Create Composite Scaffold with BMP2/COMP.

Subtask 1: Create COMP/BMP2 composites with HA/PLG

Subtask 2: Determine release kinetics and bioactivity of BMP from composite HA/PLG scaffold

What was accomplished under these goals

We had some difficulty in completing this task using the methodology originally proposed. However, we were able to use alternative approaches to accomplish the same tasks. Details are below:

Difficulty: We had originally proposed to use radiolabeled (125I iodinated) BMP2, that at the time of the proposal, was commercially available from Perkin-Elmer as (Catalog # NEX432). This represents the gold-standard for release-kinetics, since the radiolabel is small so that it does not interfere with the biological activity of the BMP2, and because radioisotope detection methods are highly sensitive and remain quantitative across a large range of concentrations. Unfortunately, this product was discontinued by Perkin-Elmer, and we were unable to identify another source of radiolabeled BMP2. A second approach would be to iodinate the BMP2 ourselves, a relatively simple procedure for which kits are readily available commercially (for example https://www.thermofisher.com/order/catalog/product/28665), and in which Drs. Haudenschild and Yik both have experience. Once incorporated into a protein, the radioactive Iodine is stable. But as free iodine, it will sublime and stick to any surface it

encounters. This property necessitates a special set of safety precautions for the iodination of proteins with free iodine. Unfortunately, the entire UC Davis campus does not have an approved facility or RUA (Radioisotope Use Authorization) for the iodination of proteins. In summary, our first approach to use radiolabeled (iodinated) BMP2 was not possible.

Difficulty: Our second fallback plan was to label the BMP2 with fluorescent tags, using methodology that has been published before by Dr. Leach's group (see PLoS ONE publication from 2011 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3187840/). In this case, exposed primary amine residues on BMP2 are labeled with an NHS-Ester activated fluorescent dye (https://www.thermofisher.com/order/catalog/product/46402) using the commercially available kit. We were successful at generating fluorescent BMP2 using this kit, but unfortunately the labeled BMP2 no longer had any biological activity as measured in our assays. We chose not to study binding or release kinetics of the fluorescently labeled but biologically inactive BMP2, because the results would be of limited relevance to our main goal of inducing bone formation.

Solution: To overcome these challenges, we chose to perform in-vitro assays to optimize the cellular responses to various formulations of BMP2 with COMP. We chose to use an established cell-based assay of osteogenic differentiation that is widely accepted, namely C2C12 cells (https://www.atcc.org/Products/All/CRL-1772.aspx#characteristics) that are cultured in base-osteogenic media. The C2C12 cell line is a mouse myoblast precursor cell that spontaneously differentiates into muscle fibers in normal growth conditions. In the presence of BMP, these cells shift their differentiation pathway from myoblastic to osteoblastic. The base-osteogenic media is permissive of osteoblastic differentiation, but by itself does not strongly induce osteogenic differentiation of the C2C12 cells. The presence of increasing amounts of BMP activity leads to a dose-dependent increase of osteogenic genes within 2-3 days, a dose-dependent increase in the production of Alkaline Phosphatase within 7-10 days, and a dose-dependent increase in the deposition of mineralized matrix within 14-21 days. These properties make the C2C12 cell culture an excellent experimental system to test for the osteogenic potency of different BMP2/COMP formulations.

Accomplishments: We determined that the optimal ratio of BMP2 to COMP that yields maximal osteogenic response in the C2C12 cells is a molar ratio of between 2:1 and 1:1 of COMP to BMP2. This is an unexpected result. Our previous observation was that each chain of the pentameric COMP can bind to one BMP, thus a maximum of 5 BMPs can be bound by each COMP pentamer. Our original hypothesis had therefore been that the most active configuration of COMP would be with all 5 BMPs bound. In effect, that COMP acts as a "BMP concentrator". However, the results detailed below consistently show that the most osteogenic formulation is with only 1 or 2 BMPs per COMP protein. A possible explanation for this might be that COMP might be most effective when it also binds to additional molecules, perhaps on the cell surface (integrins, CD47, etc.) or in the matrix (collagens, fibronectin, proteoglycans etc.). This is shown diagrammatically in Fig 1.

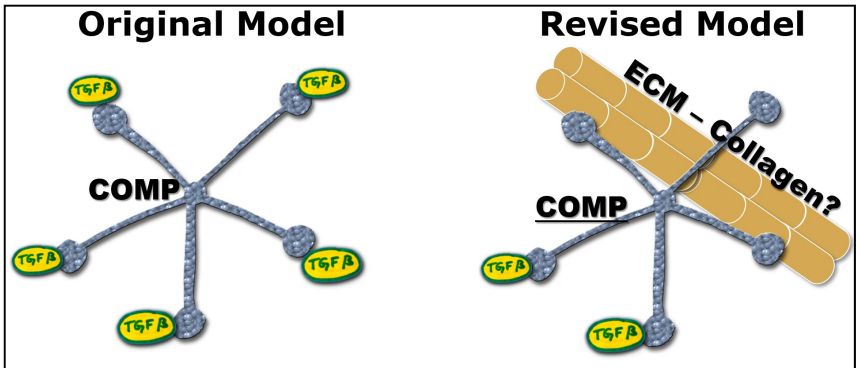

Figure 1. In the original model, we hypothesized that the biggest effect of COMP would be with a TGF-beta superfamily growth-factor (such as BMP2) bound at each binding site. However, our data suggests maximal stimulation of osteogenic activity is with a ratio of BMP:COMP that leaves some potential binding sites free to bind other extracellular or cell-surface molecules. COMP is known to also bind to collagens, fibronectin proteoglycans, integrins, CD47, complement, and more.

In-vitro Osteogenesis Optimization Experimental Approach 1: Effect of Manganese

The goal is to get the most active formulation of BMP2 with COMP, to yield the highest biological bone-forming response. This experiment was based on our published observations that the cation Manganese can increase the ability of COMP to bind to TGFb and BMP growth factors (Ishida, 2013 Bone). In fact, with TGFb we showed that manganese unveils a second growth-factor binding site, so that a maximum of ten TGFb growth factors can be bound by a single COMP (Haudenschild, 2011 JBC). It remains unknown whether this also causes an increase in the biological activity of the BMP2:COMP complex. In this experiment we tested whether binding BMP2 to COMP in the presence of manganese would have increased osteogenic activity.

The experimental system is the C2C12 myoblast cells, seeded at 1000 cells/cm2 in 6-well plates, in base-osteogenic media (alpha-MEM supplemented with 10% calf serum, beta-glycero-phosphate, 100nM dexamethasone, proline). As a negative control, we used proliferation media (DMEM with 10% calf serum), which does not induce osteogenic differentiation. For all other conditions we used base-osteogenic media. The outcome we measured was the production of alkaline phosphatase after 7 days of culture, using the substrate b-nitrophenyl-phosphate. Alkaline phosphatase is a marker of osteogenic differentiation. The control groups included base-osteogenic media alone, COMP alone (200ng/ml), or manganese alone (0.5 uM). Experimental conditions included 50ng/ml BMP2, 50ng/ml BMP2 with COMP, and 50ng/ml BMP2 with COMP and manganese (at the respective concentrations). BMP2 and COMP were allowed to pre-bind for 10 minutes in a more concentrated form, then the pre-bound proteins were added to the cell cultures to the final concentrations indicated above. Media was replenished on a M-W-F schedule, and fresh BMP, COMP, and manganese was added with each media change. Each measurement is from multiple technical replicates within the same experiment, and experiments were independently repeated at least 3 times. Statistical comparisons were performed in JMP 12.0Pro, consisting of 1-way ANOVA with Tukey's post-hoc corrections. Histogram bars not connected with the same letter are statistically different with $p<0.05$.

From these experiments, we **conclude** that COMP enhances the osteogenic response to 50ng/ml BMP2 as measured by alkaline-phosphatase activity after 7 days, but that the presence of manganese had no further effect. The results are shown in Figure 2.

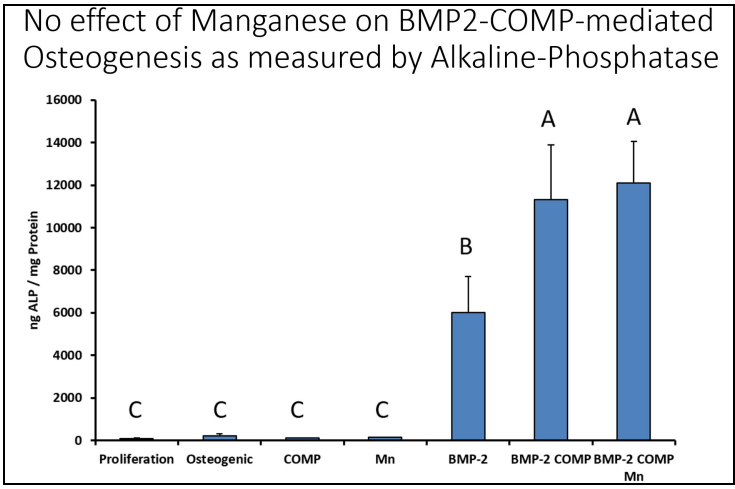

Figure 2: COMP enhances the osteogenic response to 50ng/ml BMP2 in C2C12 cells.

Manganese does not further enhance the biological response to BMP2:COMP, as measured by alkaline phosphatase activity after 7-days.

In the absence of BMP2, neither COMP nor manganese affect osteogenesis.

One-way ANOVA and Tukey's post-hoc corrections, with significance set at $p<0.05$. Histograms not connected by the same letter are significantly different

We set up an identical series of experiments to measure the deposition of mineralized matrix using a calcium-binding dye Alizarin-red. This provides an additional line of evidence for the biological response to BMP2 together with COMP and/or manganese. The methods are identical to those described above, except that the cells are cultured for 3 weeks and the amount of mineralized matrix is quantified at day 21. For analysis, the cells were briefly formalin-fixed, calcium-binding alizarin-red dye was added, and any unbound dye washed away. The bound dye was brought into solution with detergent, and the amount of soluble dye quantified by measuring the absorbance at 570nm on a 96-well ELISA plate reader.

From these experiments, we **conclude** that COMP enhances the osteogenic response to 50ng/ml BMP2 as measured by alkaline-phosphatase activity after 7 days, but that the presence of manganese had no further effect. The results are shown in Figure 3.

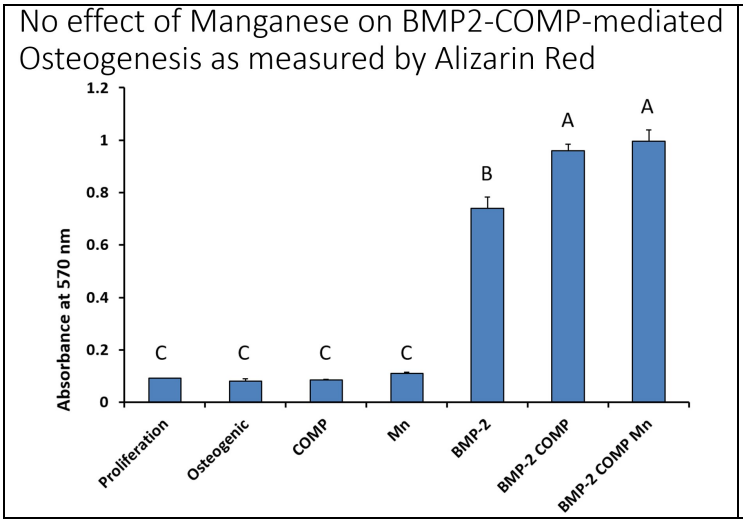

Figure 3: COMP enhances the osteogenic response to 50ng/ml BMP2 in C2C12 cells.

Manganese does not further enhance the biological response to BMP2:COMP, as measured by mineralized matrix deposition (alizarin-red) after 21-days.

In the absence of BMP2, neither COMP nor manganese affect osteogenesis.

One-way ANOVA and Tukey's post-hoc corrections, with significance set at $p<0.05$. Histograms not connected by the same letter are significantly different

In-vitro Osteogenesis Optimization Experimental Approach 2: Specificity to COMP

The goal is to get the most active formulation of BMP2 with COMP, to yield the highest biological bone-forming response. In this experiment, we tested whether the enhancing effect was specifically due to COMP protein, or a non-specific effect of having additional proteins present perhaps to prevent loss of BMP to non-specific adsorption. These experiments were performed using C2C12 cells cultured under osteogenic conditions as described above. The outcome was the activity of alkaline phosphatase after 7 days of culture in osteogenic media, with the enzymatic activity of alkaline-phosphatase measured as described above. We used casein as a source of non-specific protein, since no binding to casein has been described for BMP2 or COMP. Another often-used non-specific protein is serum albumin, but we chose not to use albumin since commercial preparations usually also contain fibronectin (a known COMP-binding protein). This would confound the interpretation of the results.

To determine whether the enhancing effect was due to specific properties of COMP, rather than non-specific effects of just having an increased protein concentration, we used a 25000-fold excess of casein (compared to COMP) as a non-specific protein. We found that 5mg/ml casein did not statistically affect the osteogenic properties of 50ng/ml BMP2 after 7 days as measured by the production of alkaline phosphatase, while 200ng of COMP with 50ng/ml

BMP2 resulted in significantly stronger osteogenic response (Figure 4). We **conclude** that the effect observed in-vitro is specific to COMP.

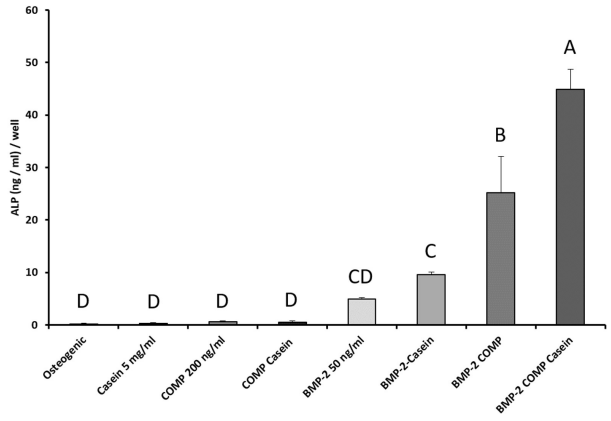

Figure 4: The enhanced osteogenesis is specific to COMP as measured by Alkaline-phosphatase activity at 7-days.

A non-specific protein (casein) does not enhance osteogenesis, even when used at a vast excess. Casein was 5mg/ml, and COMP was at 200ng/ml, representing 25,000 fold more casein than COMP.

One-way ANOVA and Tukey's post-hoc corrections, with significance set at $p<0.05$. Histograms not connected by the same letter are significantly different. Histogram height represent average values, error bars are standard deviations.

In-vitro Osteogenesis Optimization Experimental Approach 3: Determine BMP to COMP ratio

The goal is to get the most active formulation of BMP2 with COMP, to yield the highest biological bone-forming response. In this next experiment, we tested for the optimal ratio of BMP2 to COMP that would yield the most robust osteogenic response in the C2C12 cell model. Having demonstrated (in figure 4 above) that the enhanced osteogenic effect is specific to COMP and not a results of excess non-specific protein, we next set out to determine what amount of COMP most strongly enhances the osteogenic response to BMP2. Specifically, our hypothesis was that having each binding site of COMP bound to a BMP2 growth factor (BMP2:COMP ratio of 5:1) would yield the highest osteogenic activity. The approximate mass of a mature glycosylated COMP pentamer is 500kDa, and the approximate mass of the mature BMP2 growth factor is 25kDa. Using these numbers, a 1:1 molar ratio of BMP2 to COMP corresponds to a mass ratio of 1:20. The studies were performed as described above, using C2C12 cultured with base-osteogenic media. The media was supplemented with BMP2 (50ng/ml) that was pre-mixed with increasing amounts of COMP (from 1ng/ml to 1000ng/ml). Osteogenic differentiation was measured after 7 days by quantifying the alkaline-phosphatase enzymatic activity with reference to a standard curve.

We observed that COMP at 1ng/ml caused a significant increase in the amount of osteogenic activity as measured by alkaline-phosphatase production (Figure 5, next page). This represents a mixture of BMP2 and COMP in which the BMP2 is in excess, in other words, even if every BMP2-binding site of COMP were occupied there would still be excess BMP2. Increasing the amount of COMP from 0.2ng/ml to 200ng/ml (with the same amount of BMP2 held constant) showed no further increase in osteogenic activity. There appears to be a threshold of 1000ng/ml of COMP where additional COMP further enhances the osteogenic response to BMP2. Importantly, this mass ratio represents approximately a 1:1 molar ratio, a situation where (assuming 100% interaction) the average COMP is bound to 1 BMP growth factor. This experimental observation was reproducible (using the C2C12 osteogenic assay), although we have yet to validate it using an additional experimental approach. Not shown on the graph is that

COMP alone, even at the maximum concentrations of 1000ng/ml, did not induce any osteogenic response in the assay.

These results suggest that COMP is not simply a "BMP2 concentrator" that functions by binding many BMP molecules. Instead it suggests that COMP might enhance the osteogenic response by binding only 1 or 2 BMP molecules, while also simultaneously interacting with additional sites either in the extracellular matrix or on the cell-surface (as schematically diagrammed in Figure 1). Independent of mechanism, for the purposes of this project, our experimental design moving forward will use the higher amount of COMP that elicits a stronger osteogenic response. We **conclude** that approximately a 1 to 1 molecular ratio of BMP to COMP yields the most robust biological response. The corresponding mass ratio of 50ng BMP per 1000ng COMP will be used for future experiments.

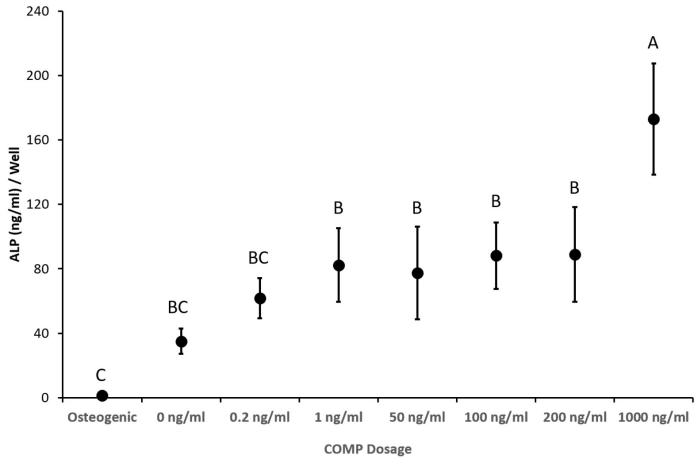

Figure 5: Higher concentrations of COMP further increase the osteogenic response.

The addition of increasing amounts of COMP (on the X-axis) enhances the osteogenic response to a fixed amount of BMP2.

There appears to be a plateau in the dose-response between 0.2ng/ml and 200ng/ml of COMP. However, a further increase in osteogenic response is seen with 1000 and 2000 (not shown) ng/ml of COMP. At these amounts, the molecular ratio of BMP:COMP is approximately equal.

One-way ANOVA and Tukey's post-hoc corrections, with significance set at $p<0.05$. Values not connected by the same letter are significantly different. Points represent average values, error bars are standard deviations.

What opportunities for training and professional development has the project provided?

The project includes one graduate student, Michael Fong, who is a PhD candidate in Biomedical Engineering. I made a mistake in the budget for Michael, I only included a line item for tuition and fees, but not for his salary. So he has had to support himself through opportunities as a teaching assistant for his salary through December 31, 2016. As of January 2017, he is full-time dedicated (no TA) to this project, and his productivity has been greatly increased as a result. Michael has presented his work on this project at internal group lab meetings, and is scheduled to give a formal lecture on the topic to the UC Davis' Veterinary Orthopedic Research Seminar Series on February 23th, 2017.

How were the Results disseminated to communities of interest?

The results are a part of an abstract accepted for presentation as a poster at the annual meeting of the Orthopaedic Research Society to be held in San Diego this spring. PhD candidate Michael Fong is presenting a seminar based in part on this project at the to the UC Davis' Veterinary Orthopedic Research Seminar Series on February 23th, 2017.

What do you plan to do during the next reporting period to accomplish the goals?
We established the formulation for BMP2 and COMP that has maximal biological activity in our in-vitro assays of osteogenesis. The next step is to validate that this formulation induces bone formation with the HA/PLG scaffold, first in-vitro, and then in the femoral defect model in-vivo. IACUC protocols are being finalized, and will be submitted to ACURO for review. Due to the unexpected delays from the discontinued 125I-labeled BMP2 product and the TA-duties of Michael Fong, we expect to request a no-cost extension for the completion of the work.

IMPACT

What was the impact on the development of the principal discipline(s) of the project?
The major finding was the observation that fully saturating all of the BMP-binding sites of COMP with BMP does not optimally enhance the biological response as measured by osteogenic differentiation of C2C12 cells. This was unexpected. For basic research, this observation opens up new areas of research to further discover the exact mechanisms of action. For translational research, this observation will guide the formulation of BMP2:COMP for the remainder of the experiments. Pragmatically, the impact is that we have to scale up our production of recombinant COMP protein.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
We filed a patent on the use of COMP to bind growth factors, see US Patent US 9133259 B2 "Cartilage oligomeric matrix protein (COMP)—growth factor complexes and uses thereof." This patent was granted to UC Davis in late 2015. It may be possible to build on this Use patent with a Formulation patent based on the new observations about the ratio of BMP to COMP that yields the maximal osteogenic response. We will discuss this possibility with our tech transfer office.

What was the impact on Society beyond science and technology?
Nothing to Report.

CHANGES / PROBLEMS

Changes in approach and reasons for change
As detailed above, the ^{125}I radiolabeled BMP2 that we proposed to use for binding kinetics studies in Aim 1 is no longer produced, no substitute exists commercially from the original vendor or other vendors. Furthermore, at UC Davis (the entire campus) we no longer have the ability to create this reagent in-house. Alternative approaches using fluorescent labeling techniques were attempted, but these larger labels decreased the biological activity of the growth factors. This necessitated a shift in the approach away from determining the in-vitro binding

kinetic (as originally proposed). Our revised approach focused on maximizing the biological response to a fixed amount of BMP2. While the biological activity studies were also integrated as part of the original proposal, we have expanded their scope considerably given that the binding kinetics were no longer possible. Ultimately, the biological activity is what matters for clinical translation, so we feel that this change is beneficial to the overall goals of the research.

Actual or anticipated problems or delays and actions or plans to resolve them

I mis-budgeted the salary and fees for PhD candidate Michael Fong. This resulted in delays, as Michael had to support his salary through teaching assistant positions until January 2017, rather than work full-time on this project. As of January, he is working full time on this project, with a corresponding increase in overall productivity and progress.

Changes that had a significant impact on expenditures

A substantial portion of the budget is still available due to delays in starting the project. We encountered delays in attempting to locate a commercial source of ^{125}I labeled BMP2, and further delays in attempting to get approval for generating the reagent in-house. Another time set-back was that the fluorescently labeled BMP2 did not have biological activity, so we decided to not perform release-kinetics studies with a labeled but inactive growth factor. Finally, there was a delay in assigning full-time effort to the project for Michael Fong. The result of these delays are that we are behind schedule for the expensive animal studies, but with an appropriate amount of the budget still available. We expect to request a no-cost extension for the completion of the project.

Significant changes in the use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

A delay in IACUC and ACURO protocol submission was caused by the delay in the research as outlined above. Now that we have identified a formulation of BMP2 and COMP with maximal biological activity, we are finalizing the IACUC protocol with ACURO submission to follow. No animal studies will be performed until we obtain both IACUC and ACURO approval.

PRODUCTS

Publications, Conference Papers, and Presentations

Journal Publications

– Nothing to report

Books or other non-periodical, one-time publications

– Nothing to report

Other Publications, conference papers, and presentations

– In 2016, results were presented at the 2016 American Chemical Society's meeting in San Diego, by PhD student Victoria Tran (the work included recombinant COMP protein made with funds from this grant). An abstract is included at the end of this report.
– In 2017, results will be presented by PhD candidate Michael Fong at the Annual Meeting of the Orthopaedic Research Society in San Diego. An abstract is included at the end of this report.

Website(s) or other internet sites

– I occasionally update my lab's WordPress website (http://lab.haudenschild.org) with news about the lab. With each update the WordPress script then automatically updates the lab's Facebook page (https://www.facebook.com/Haudenschild.Research/), the lab's Twitter feed (https://twitter.com/DHaudenschild), and my personal LinkedIn profile (https://www.linkedin.com/in/haudenschild/). UC Davis recently made WordPress available for faculty, and I'm in the process of migrating my own site (privately hosted at my expense) (http://lab.haudenschild.org) onto the University's servers, which will be at (http://orthoresearch.faculty.ucdavis.edu/).

Technologies or Techniques

– Nothing to report

Inventions, patent applications, or licenses

Nothing new. Based on preliminary data that went into this grant proposal, we were granted a patent "Cartilage oligomeric matrix protein (COMP)—growth factor complexes and uses thereof" in 2015.

Other Products

- Data or databases; None
- Biospecimen collections; none
- Audio or video products; none
- Software; none
- Models; none
- Educational aids or curricula; none
- Instruments or equipment; none
- Research material; We scaled up the production of recombinant human COMP protein, and have used some of this protein to initiate new collaborations
- Clinical interventions; none
- New business creation; none
- Other; none

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on this project

No change

Has there been a change in the active or other support of the PD/PI or senior/key personnel since the last reporting period?

One previously active grant ended. This grant had provided salary support for both PI (Dominik Haudenschild) and co-investigator (Jasper Yik). The grant that ended has no overlap (scientific or effort) with the current grant.

Early Intervention with CDK9 Inhibitors to Prevent Post-Traumatic Osteoarthritis
PRMRP IIRA PR110507 (PI: Haudenschild) 10/1/2012 – 9/30/2016
The goal of this project is to prevent the onset and progression of OA after traumatic joint injury, by interfering with primary response gene transcriptional activation specifically through inhibition of Cdk9. There is no scientific or effort overlap with the current grant.
Role: PI

One new NIH grant application scored well, and we're hoping that it will be funded. This will support both PI (Haudenschild) and co-investigator (Yik). The topic of this new grant is related to the current proposal, in that the goals are to further investigate at a very basic science level how the multivalent presentation of growth factors affects growth factor activity. We were careful avoid any overlap with the current proposal.

Multivalent Presentation of Growth Factors Regulates Cellular Responses
NIH R01 AR070239 (PI: Haudenschild) 4/1/2017 – 3/31/22
The goal of this project is to determine the fundamental mechanisms of how the multivalent presentation of growth factors affects the cellular responses. We use the COMP/TGFb and COMP/BMP interactions as model systems, and explore possible mechanisms such as receptor-co-localization, substrate turnover, binding to additional matrix components, and more. There is no scientific or effort overlap with the current grant.
Role: PI.

What other organizations were involved as partners
Nothing to report

SPECIAL REPORTING REQUIREMENTS

Collaborative Awards
Not applicable

Quad Charts
Not applicable

APPENDICES

2 appendices are attached:

1. The ACS abstract presented in 2016
2. The ORS abstract that will be presented this spring

Title: **Multivalent presentation enhances the evolution of membrane structure and actin assembly of C2C12 cells**

Authors: Victoria Tran[1], Arpad Karsai[1], Michael Fong[2], Evgeny Ogorodnik[1], Jasper Yip[2], Dominik Haudenschild*[2], Gang-yu Liu*[1]

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Abstract

Bone morphogenetic protein-2 (BMP2), a member of the transforming growth factor-β family, is known to induce osteogenic differentiation among chondrocytes, osteoprogenitor cells, and human mesenchymal stem cells. Pentameric cartilage oligomerization matrix protein (COMP) binds BMP2 and presents it to the receptors of C2C12 cells. Our prior investigation indicates that the COMP+BMP2 complex enhances the activity of BMP2 in the context of osteogenic differentiation via the Smad pathway. This presentation provides a more molecular level and single cell level look into the mechanism of this enhancement. Using multimodal and multifunctional approach of atomic force microscopy image, single cell mechanics, and confocal imaging, our investigation reveals that treatment of COMP+BMP2 to C2C12 increases the stiffness of the cell membrane and appearance of fiber-like features on the cells. Strong presence of actin stress fibers at the basal interface of the cells and the rearrangement and formation of ordered actin filaments on the apical surface are evident compared to cells treated with BMP2. These findings help us understand the mechanism of this enhancement, therefore, helping us devise an optimally doses of BMP and COMP to stimulate bone regeneration.

Multivalent Presentation of Growth Factors by Cartilage Oligomeric Matrix Protein

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Introduction: The purpose of this work is to demonstrate that the presentation of growth factors (GFs) by COMP enhances the growth factor efficacy for osteochondral tissue engineering. Growth factors including bone morphogenetic protein (BMP) and transforming growth factor beta (TGFB) have tremendous clinical utility in forming bone and engineered cartilage tissues. It is common knowledge that these growth factors require binding to extracellular matrix (ECM) proteins for their full activity on cells. However, the mechanisms through which these GF/ECM binding interactions enhance growth-factor activity remain largely unknown. Cartilage Oligomeric Matrix Protein (COMP) is a homo-pentameric extracellular protein abundant in cartilage and bone. We recently discovered that COMP binds to BMPs and TGFB growth factors. Importantly, this binding enhances growth factor activities both in-vitro and in-vivo. COMP enhances the osteogenic activity of BMP2 in a rat model of spinal fusion, and COMP enhances the chondrogenic activity of TGFB1 for cartilage tissue engineering. Previous work by others established that many COMP binding interactions require zinc and manganese, two trace-elements that are absent in serum-free cell culture media. Here we explore the effects of these trace elements on osteochondral tissue engineering in the presence of COMP. We believe that enhancing COMP's ability to bind with the matrix by adding zinc may further enhance COMP's ability to present growth factor to receptors and enhance the chondrocyte phenotype.

Methods: Human cartilage samples were acquired from consented patients undergoing TKA or THA surgeries at our institution, with IRB approval. Chondrocytes from different donors were either extracted from cartilage by collagenous digest or supplied as clinical grade autologous cultured chondrocytes. Chondrocytes were expanded in monolayer to passage 3 maximum. Cell viability in the presence of zinc sulfate (0-320uM) was measured by WST-1 assay in monolayer cultures of chondrocytes after 2 days. Chondrogenic pellet culture was with $4x10^5$ cells per well of 96-well conical-bottom non-adherent culture plates, centrifuged 350xg for 5 min. Pellets were cultured for 3 weeks with media changes every other day in chondrogenic base media of 50 ug/ml ascorbic acid, 40 ug/ml L-Proline, 100 nm dexamethasone, 1% ITS, 15mM HEPES, and 10 ng/ml TGFB-1 and supplemented with zinc sulfate and/or COMP from 2-200uM or 0.1 – 15 µg/ml, respectively. After 3 weeks, pellets were weighed to obtain wet weight (ww) and imaged, and glycosaminoglycan (GAG) content measured by DMMB assay. Data from was analyzed with ANOVA with Tukey's HSD test. Human 293T cells were stably transfected to over-express recombinant human COMP with a C-terminal HisTag. COMP was extracted from the media by nickel-affinity chromatography, resulting in >95% purity by SDS-PAGE. Purified COMP was imaged with atomic force microscopy to visualize the expected pentameric structure.

Results: Atomic Force Microscopy revealed that the recombinant COMP was pure, and primarily in pentameric form (Figure 1). The addition of Zinc was non-toxic to monolayer chondrocytes, up to 320 uM (Figure 2A). In TGFB-induced chondrogenic pellet culture, zinc increased the pellets wet weight or GAG/ww ratio, with an optimal zinc concentration of 50 uM (Figure 2B). Above 50 uM zinc, the pellets were of inferior quality, soft and gelatinous (data not shown). We therefore used 50uM zinc for the remaining experiments. COMP also enhanced the wet weight of the pellet cultures, either alone or in combination with 50 uM zinc (Figure 3). The enhancing effect of COMP did not appear to be additive with that of zinc. Although COMP and zinc consistently enhanced TGFB-induced in-vitro chondrogenesis in all donors tested, there was variability between donors. In some donors the effects were most apparent in pellet size, wet weight, and total GAG content, whereas in other donors the effects were most apparent in the GAG/wet weight measurements. One possible explanation may be donor-dependent proliferation rates of the isolated chondrocytes, but this remains to be proven.

Discussion: We demonstrated that growth factor presented on COMP presents enhances the growth factor activity. COMP also other extracellular matrix elements such as chondroitin sulfate and collagen, and many of these interactions are cation-dependent. Perhaps these cation-dependent interactions are also with other ECM elements are important for the enhancing effect of COMP on growth factors that we observe. Zinc is an ion not typically present in the serum-free and chemically defined media used for tissue engineering. Therefore, the effects of zinc on chondrocyte pellet culture was determined. Previous studies demonstrate that zinc enables a more effective COMP-collagen interaction, which would help to tether COMP in close proximity to the cells while presenting TGFB-1 to the receptors. While the number of donors is limited in these preliminary experiments, these results highlight simple and novel additions to culture technique that may increase the efficacy of current orthopedic therapy and approaches the production of engineered osteochondral tissues.

Significance: COMP and divalent cations may have clinical utility to more effectively fill defects or maintain a better chondrocyte phenotype.

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Figure 1

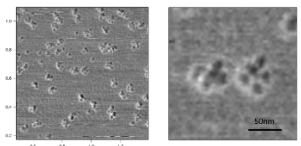

Figure 2

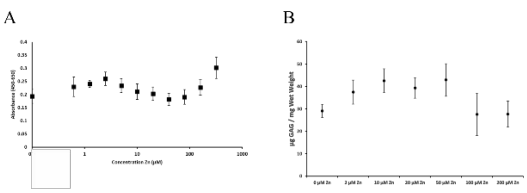

Figure 3