

AD_____

Award Number: W81XWH-12-1-0274

TITLE: Diagnosis and Treatment of Heterotopic Ossification

PRINCIPAL INVESTIGATOR: Dr. Alan R. Davis

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, TX 77030

REPORT DATE: October 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation \

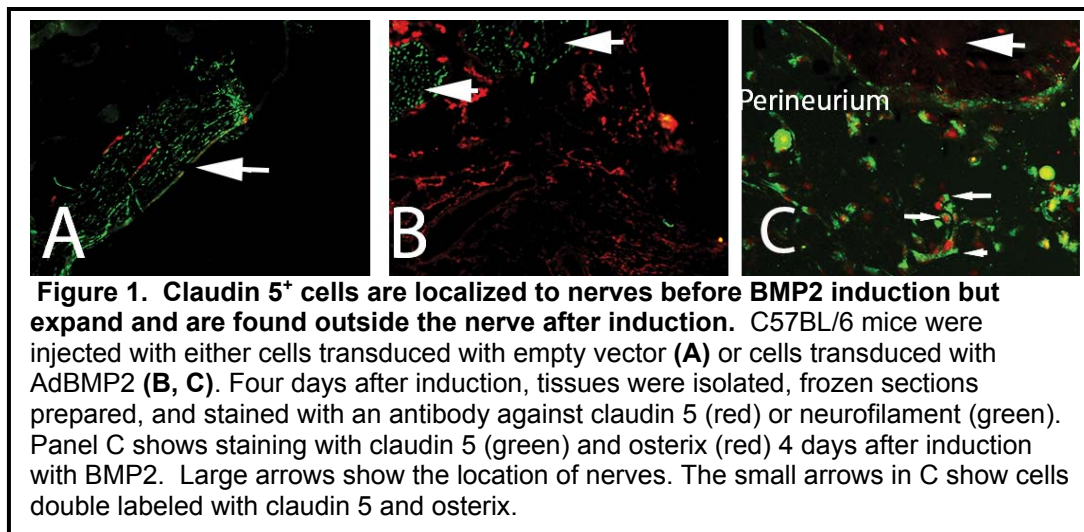
REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE October 2013		2. REPORT TYPE Annual		3. DATES COVERED 30 September 2012 - 29 September 2013	
4. TITLE AND SUBTITLE Diagnosis and Treatment of Heterotopic Ossification				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-12-1-0274	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Alan R. Davis and Dr. Elizabeth A. Olmsted-Davis E-Mail: ardavis@bcm.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Baylor College of Medicine Houston, TX 77030				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT We recently developed a model of heterotopic ossification (HO) that suggests blocking the initial response of the nerve could ultimately block this devastating problem before it occurs. Studies proposed in this application to identify and isolate osterix ⁺ cells have led us to identify a second tentative progenitor within peripheral nerves. These cells reside in the endoneurial compartment and express Claudin 5, PDGFR α , and osterix but are negative for the Schwann cell marker P75 and perineurial marker Claudin 1. We have isolated the Claudin 5 ⁺ , PDGFR ⁺ and negative populations and confirmed that 100% of the osterix expression co-purified with the claudin 5 ⁺ population. Claudin 5 is present on specialized endothelial cells within the endoneurium and is responsible for forming the tight junction of blood nerve interface. We are further characterizing these cells for expression of endothelial markers. We also looked at the expression of Wnt1, a marker of neural crest stem cells. Our preliminary data suggests that these cells undergo significant expansion after induction of HO; however, they remained localized within the peripheral nerves. However, 100% of the Wnt1 ⁺ cells appeared to be also claudin 5 ⁺ suggesting that the Wnt1 cells may be precursors to a more differentiated progenitors. We are currently following up on these and other experiments proposed in the application.					
15. SUBJECT TERMS BMP2, Heterotopic ossification, endoneurial cells.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)
			UU	9	

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusion.....	8
References.....	9
Appendices.....	9

INTRODUCTION: Heterotopic ossification (HO), the formation of bone in the muscle or other soft tissue, or any non-skeletal site can cause severe problems of pain and disability. It often requires the patient to undergo additional surgery. A particularly frustrating problem in amputees is the growth of bone within the amputation stump, making prosthesis wear difficult or impossible. Such heterotopic bone also develops spontaneously near the joints in many patients with an injured spinal cord. Tentative inhibitors, such as low dose radiation that have some efficacy in preventing HO in patients at high risk, cannot be implemented in the majority of cases. Thus there are currently no available efficacious treatments. Although the incidence of HO in the general population is fairly low, approximately 11%, it is a significant problem within the military population where the incidence is approximately 60-70% of all traumatic injuries. Here we present data to suggest that in actuality the primary source of HO is the peripheral nervous system (PNS). We recently identified one of the earliest steps in HO is the remodeling of the nerve structure through a key process induced by BMP2. **We hypothesize that the regulation of heterotopic ossification may be induced through neurogenic inflammation and peripheral nerve signaling, which leads to remodeling of the nerve and release of osteoprogenitors.** Here we propose that remodeling of the nerve itself, leads to the release of stem/progenitors from the nerve, which contribute to the structures that make up bone, including osteoblasts. We propose to characterize these progenitors, demonstrate their functional role in HO, and utilize the mechanism of this process for the development of a blood test for early detection. Knowledge of mechanism also specifies molecular targets for design of agents to treat HO.

BODY: Task 1: To isolate and characterize the nerve stem/progenitor population: Since their original description in 1999 [1] bone marrow mesenchymal stem cells have been thought of as the progenitor for osteoblasts during HO [1,2,3]; however, recent work suggests that there may be a local stem/progenitor cell [4]-[5]. Using our model of HO, we recently identified the rapid expansion of a progenitor within the endoneurium of peripheral nerves that expressed the stem cell markers *nanog* and *klf4*, as well as *osterix*, a transcription factor that regulates osteogenesis [6]. The endoneurium of peripheral nerves houses the axons and Schwann cells. This compartment is separated from the external environment by the perineurium, which functions, along with the endoneurial vasculature, as a barrier [7]. We next attempted to determine whether *osterix* expression within the endoneurium was associated with one of the known cell populations that reside in this region.



Immunostaining the tissues for the Schwann cell markers (proteolipid protein 1 and nerve growth factor receptor, P75) showed that they did not coincide (data not shown). Recently Yosef *et al* [7] reported the unique expression of the tight junction molecule, claudin 5, on specialized endoneurial endothelial cells (EECs) and suggested it plays a critical role in regulating

the blood-nerve interface similar to that observed in the brain where claudin 5 (^{-/-}) mice do not survive because of the lack of an effective blood brain barrier [8]. Furthermore even though many ECs express claudin 5 under diverse circumstances [9], it is only expressed in peripheral nerves in the mouse hind-limb tissues prior to induction of HO (Figure 1). Immunohistochemical staining for claudin 5 expression in mouse hind-limb shows the presence of a handful of cells within the endoneurium of peripheral nerves (as detected by neurofilament H) (Figure 1, panel A), but absent from any other tissue structures including the normal blood vasculature (Figure 1, panel A). However four days after induction of HO, these claudin 5⁺ cells can now be seen both inside and outside of the nerve structures, and in between muscle fibers, which reside between peripheral nerves and the site of new bone formation (Figure 1, panel B).

We next determined whether these cells were co-localizing with the *osterix*⁺ cells in the nerve. *Osterix* appeared to co-localize to the nucleus of claudin 5⁺ cells (Figure 3, Panel C). From these initial immunostains, we cannot conclude that these cells are migrating away from the nerve towards HO, but the expression pattern

involves the area that is between the reacting nerves and new bone, which is highly suggestive that the nerve structure is involved in this process.

We next quantified the number the claudin 5⁺ cells through fluorescence activated cell sorting (FACS). In these experiments, soft tissues were isolated at time 0, 2, and 4 days after induction of HO, collagenase digested, and cells subjected to cell sorting. The results (Figure 2) confirm that claudin 5⁺ cells increase almost 10-fold as early as 2 days after induction of HO, but then decline somewhat by day 4, although still significantly elevated over time 0 (or control). This may reflect the initial expansion of these EECs and differentiation to osteoblasts. We next confirmed the co-expression of claudin 5 and osterix, by immunostaining the isolated cells. Both the claudin⁺ and - populations (green) were collected and cytospun onto slides, which were further immunostained for osterix (red) (Figure 3). Cells were counterstained with DAPI (blue). The majority (approximately 75%) of the claudin 5⁺ population also expressed osterix (panel B and E). As seen in panel C, and F, when we overlayed the images, we observed some osterix⁺ cells that were very weakly positive for claudin 5 and some claudin 5⁺ cells that were not expressing osterix, suggesting that the cells may be down regulating claudin 5 as they up-regulate osterix. The claudin 5⁻ cells, figure 5, panels G, H and I, had similar numbers of cells as the positive population (Panel I), but we observed no positive staining for osterix, panel H and I.

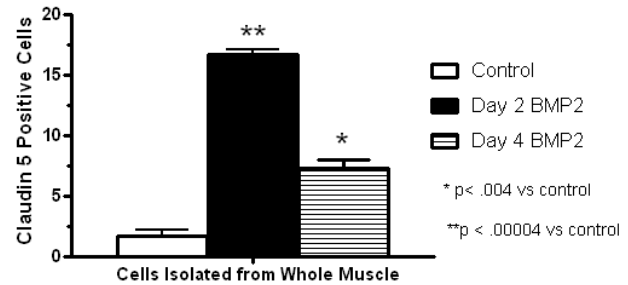


Figure 2: Increase in claudin 5 positive cells in muscle two and four days after BMP2 induction. C57BL/6 mice (n=18) were either injected with Ad5BMP2-producing cells or remain untreated. At two and four days after BMP2 induction three mice from the BMP2-induced group and three controls were euthanized and the cells released from whole muscle around the site of injection by protease digestion. Cells were subjected to FACS using an antibody against claudin 5 and the percentage of claudin 5 positive cells was determined.

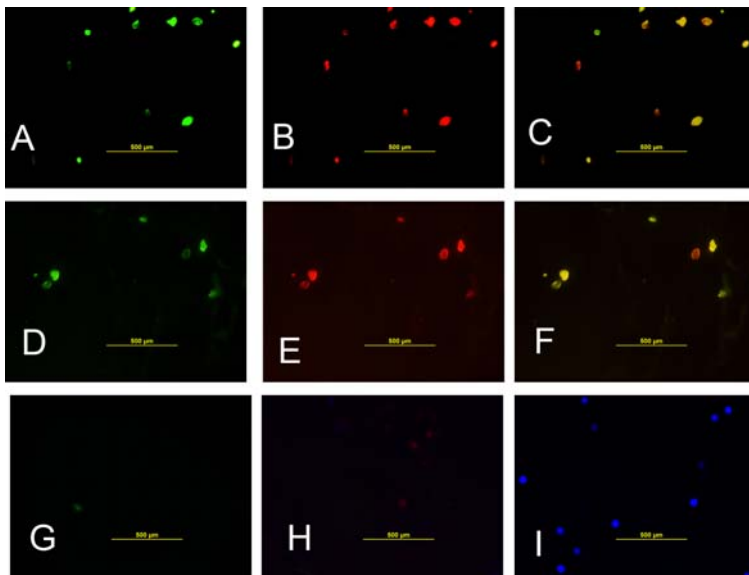


Figure 3: Immunocytochemistry for osterix shows co-localization with claudin 5⁺ cells. Claudin 5 positive (green; panels A and D) and negative cells (panel G; no staining) was isolated using FACS sorting of cells from soft tissues in the hind-limb 4 days after induction of HO. Both populations were cytospun onto slides and immunostained for osterix (red; panels B, E, and H). Two separate representative fields are shown for the claudin 5⁺ populations. Panels A and B are merged in C; panels D, E are merged in panel F. In the claudin5⁻ population (panel G) there is a lack of osterix⁺ staining (panel H, red) although there are similar numbers of cells on the slide as compared to the claudin 5⁺ population slide, as determined by counterstaining the cells with DAPI (panel I).

We next looked at the expression of PDGFR α in these tissues. Previous reports suggest that PDGFR α is expressed only in primitive endoderm in early embryogenesis and then during organogenesis, in regions of epithelial and mesenchymal interaction, such as the tooth bud and bronchi and mesodermal derivatives, the lens, apical ectodermal ridge, glial precursors, cardiac valves, and choroid plexus [10]. FACS revealed that 95% of the claudin 5⁺ cells also expressed PDGFR α , and 75% of the PDGFR α cells were double positive. Further, there was a significant increase in these cells during HO (Figure 4), suggesting that our tentative endoneurial endothelial-like progenitors may be similar to those osteogenic precursors previously described [4].

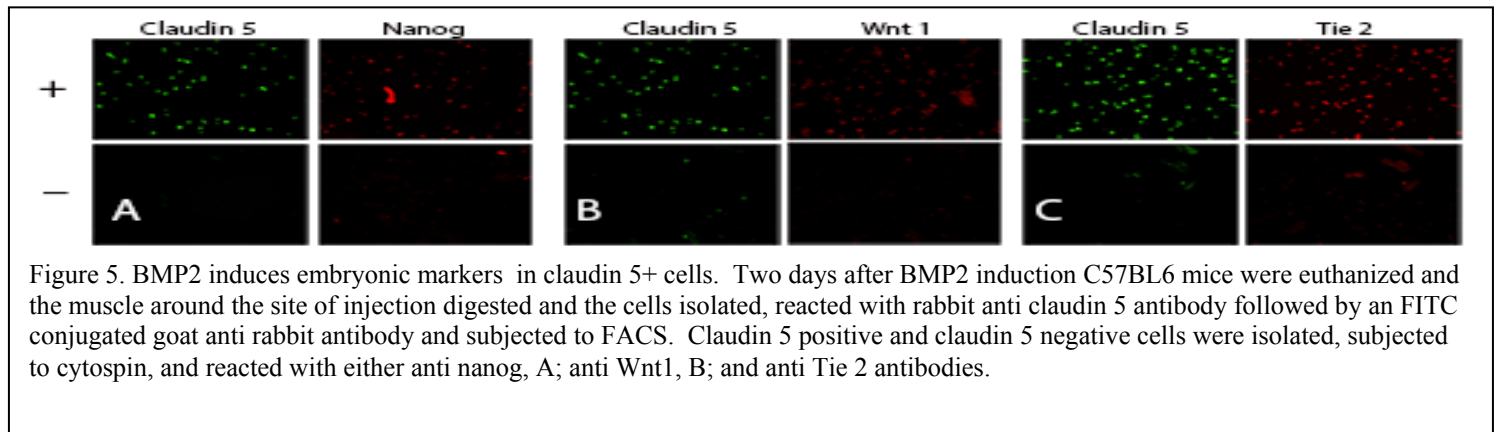
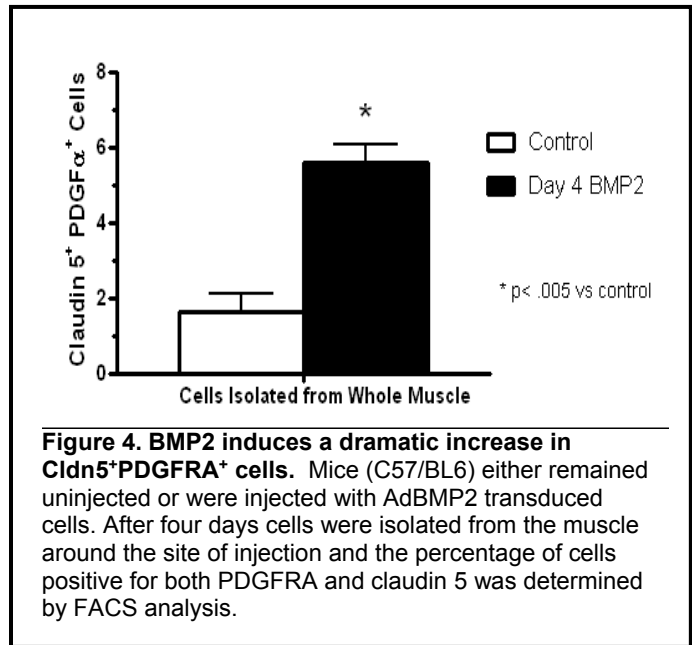
Interestingly, previous reports demonstrated that the potential endothelial-like progenitor does not arise from blood vasculature within the muscle fibers [4]. Thus we hypothesized that it is highly likely that this cell is derived from the nerve. However, suppression of nerve remodeling did not result in suppression of their expansion (figure 5). In these studies, we suppressed nerve remodeling via

neuroinflammation through delivery of cromolyn [6]. FACS analysis of the whole tissues isolated 4 days after the induction of HO, in the presence of cromolyn or vehicle, showed a similar expansion of these cells.

However, it is unclear whether the claudin 5⁺ cell expansion in the presence of cromolyn, shows the same pattern as in figure 1, or whether they are entirely within peripheral nerves, as previously reported for osterix [6]. We have generated tissue sections from one of the replicate animals in these studies, and are currently immunostaining for the presence of UCP1 (which should be absent) and claudin 5.

Since we previously showed that the osterix⁺ cells also expressed stem cell markers (nanog and Klf4), we next determined whether these cells also expressed Wnt1. Wnt1 is critical in the induction and migration of neural crest stem cells in the embryo [11] [12]. In addition, it has been suggested as a key factor in the epithelial to mesenchymal transition [13] as well as a surprisingly important factor in angiogenesis [14]. Since endothelial progenitors were recently reported to undergo an EMT in HO we questioned whether these EECs could be derived from a Wnt1⁺ neural crest progenitor.

We also analyzed the claudin 5⁺ cells for the embryonic markers nanog and Wnt1 as well as the endothelial marker Tie2. Figure 5 shows that each of these markers are present in the claudin 5⁺ but not the claudin 5⁻ negative cell populations. We consider it likely that the claudin 5⁺ endoneurial endothelial-like cells is indeed a progenitor for the osteoblast. Indeed Wnt1 [15], nanog [16] and Tie2 [17] have been reported by others to be expressed in osteoprogenitors using various methodologies.



Task 2: To identify the functional contribution of these stem/progenitors to heterotopic ossification.

To test whether the tentative osterix⁺ cells are contributing to osteoblast populations in HO, we employed a lineage tracing mouse (Ert-Wnt-YFP), which permanently activates YFP expression when the Wnt1 promoter is activated in the presence of tamoxifen. Mice were pre-treated with tamoxifen or vehicle, and then tissues isolated 4 days

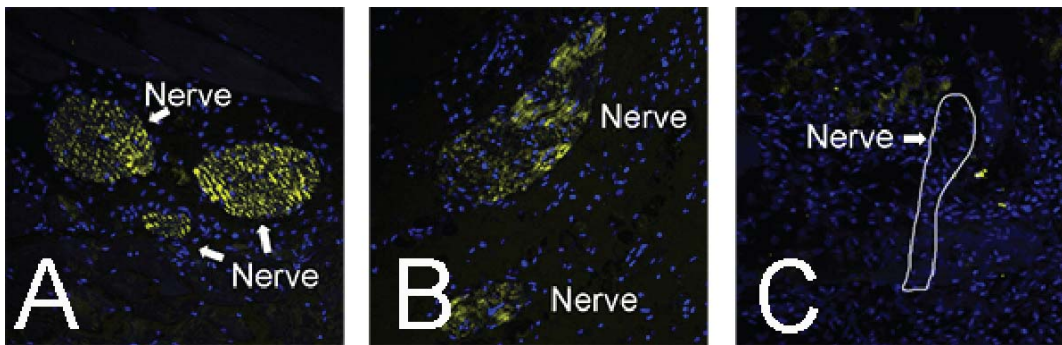


Figure 6. Expression of Wnt1-YFP in tissues isolated 4 days after induction of bone formation. ERT-Wnt1-YFP mice (n=4 per group) were treated with tamoxifen for 5 days. HO was then induced in two of the groups (A) and (B) and after 4 days mice were euthanized, sections prepared, and YFP visualized by confocal microscopy (blue is DAPI). In (B) bone formation was induced 2 weeks after delivery of the tamoxifen.

after induction of HO to determine if the claudin 5⁺ cells co-localized with the YFP label (Figure 6). YFP expression was observed within the nerves 4 days after induction of HO, but absent in nerves in the control, suggesting that Wnt1 expression had been upregulated by BMP2. However we did not observe many cells outside the nerve, suggesting that the Wnt1 labeled cells, may be repopulating tentative nerve progenitors, rather than co-localizing with claudin 5⁺ cells. We also performed preliminary FACS analysis of cells isolated from these animals, and found that only a minor percentage of the claudin 5⁺ cells co-expressed Wnt1 (data not shown). Thus the expansion of Wnt1⁺ cells suggests that they may be an earlier stem/progenitor than the claudin 5⁺ osterix⁺ cells tentatively migrating from the nerve.

Therefore to confirm this hypothesis and demonstrate a nerve origin of the claudin 5⁺ cells, we propose a prolonged treatment with tamoxifen to “chase” intermediate progenitors and allow usage of early Wnt 1 positive ones. To do this the mice will be exposed to tamoxifen at the time of weaning, so as to provide additional time for cellular attrition, and labeling of more differentiated cells derived from the Wnt1⁺ cell. Secondly, we will induce neurinflammation in the absence of BMP2, to allow for nerve remodeling, and repopulation of the progenitors from the stem cells expressing YFP. This will be done several weeks prior to inducing HO and then we will track the YFP lineage marker to determine if we now see the YFP within osteoblast populations.

We also proposed in this aim to test the functionality of these osterix⁺ cells through transplantation of a portion of the nerve from an osteocalcin-cre x R26R-YFP mouse into a wild type animal, and then follow whether we observe YFP⁺ cells within bone. Since the surgical defect in the nerve created through the transplantation experiment will potentially result in an altered environment, we propose to take a different approach to definitively test whether these osterix⁺ cells are contributing directly to osteoblasts in HO. Our proposed approach will be to cross mice which have the endogenous osterix gene flanked with lox P sites, so that in the presence of cre, osterix expression will be ablated. We will therefore cross the tamoxifen regulated Wnt1-Cre and/or PDGFR α -Cre into these mice, so that only the cells expressing either wnt1 or PDGFR α will be ablated for osterix. Therefore, we can induce HO in these mice, and characterize the resultant bone formation as to the number of osteoblasts, bone volume, density, and other parameters. We predict that since osterix is a master transcription factor for osteoblasts, that selective ablation in these cells, will disrupt bone formation during HO, if they are directly utilized as progenitors. Our collaborator Dr. Benoit de Crombrughe has agreed to provide us his previously generated and characterized floxed osterix mice.

Task 3: Identification of key changes in the blood profiles that may represent a potential biomarker for HO. We currently haven't started these experiments, but are in the process of setting up the cytokine bead arrays that will provide data for to support the essential role of neuroinflammation in this process. Further, we are also measuring factors correlating to the M1 and M2 inflammatory pathways, since neuroinflammation is an M2 pathway, which suggests then that M1 pathways will be suppressed. These studies are a major focus for the upcoming year, and we predict that in the next year we will have confirmed our hypothesis, and identified several cytokines that change during HO, in our rodent model. From there we will attempt to expand our collaboration with Dr. Jonathon Forsberg at Walter Reed to look at these factors in human tissues that represent various stages of HO. A fully executed Cooperative Research and Development Agreement between

the Department of the Navy and Baylor College of Medicine is now in place so that these studies can be carried out.

KEY RESEARCH ACCOMPLISHMENTS:

- Approval of animal experiments
- Confirmation of the osterix expression in cells within the endoneurium of peripheral nerves.
- Demonstration that the osterix⁺ cells also express the unique tight junction molecule claudin 5.
- Demonstration that the osterix⁺ claudin 5⁺ cells also express PDGFR α , which is not associated with normal blood vasculature.
- Demonstrated that suppression of nerve remodeling through delivery of cromolyn, did not suppress the expansion of the claudin 5⁺ cells; although we have previously shown that this suppress bone formation, through suppression of perineurial cells expansion.
- Demonstrated the rapid increase in Wnt1 during heterotopic ossification, which appeared to co-express claudin 5. However, the majority of claudin 5 cells did not express the Wnt1 lineage tag, thus suggesting that the osterix⁺ cell is most likely a progenitor rather than primitive stem cell.
- We are currently performing experiments to pre-label the tentative endoneurial progenitors with the lineage tag, through inducing neuroinflammation in the absence of bone formation. Then we will induce bone formation and analyze the resultant tissues for the YFP tag.
- We are currently performing experiments to introduce the tamoxifen regulated Wnt1-cre and PDGFR α -Cre into mice with two floxed osterix alleles. Once we have obtained these mice we will induce HO, and analyze the resultant bone formation to validate or negate our hypothesis that endoneurial cells within peripheral nerves directly contribute to osteoblasts during HO.
- We have ongoing studies to identify and investigate the role of neuro-inflammation in this process. We are currently developing and obtaining bead arrays to look at specific cytokines within the mouse circulation to determine if we can detect changes or a signature associated with the induction of HO.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

Salisbury E.A., Lazard Z.W., Ubogu E.E., Olmsted-Davis E.A., Davis A.R. Astrocyte-like cells from the peripheral nerve generate brown adipocytes and contribute to endochondral bone formation. International Bone and Mineral Society 43rd International Sun Valley Workshop: Musculoskeletal Biology, Sun Valley, ID, Aug 4-7, 2013- *oral presentation* (Selected for a Young Investigator Award)

CONCLUSION: We have identified a cell population in the endoneurium of peripheral nerves that expand during heterotopic ossification (HO). Because of its expression of the unique tight junction molecule claudin 5 we consider this cell to be endothelial or endothelial-like, but we are currently confirming its endothelial nature through additional immunostaining for Von Willibrand Factor (VWF) and PECAM (CD31). We have shown that these cells rapidly expand 10-fold within 48 hours of induction of HO, and then start to decrease by 4 days after induction through fluorescence activated cell sorting. Osterix expression was analyzed in the isolated claudin 5⁺ and - populations and results demonstrated that it co-purified with the claudin 5-containing population. We further, have shown that these cells also express the previously reported osteogenic marker PDGFR α , which is not expressed on normal blood vasculature, but associated with the nervous system. We found that the expansion was not suppressed by cromolyn, which we previously have shown suppressed expansion of perineurial progenitors [18]. These results suggest that remodeling of the epineurial matrix and expansion of the perineurial cells may not be a pre-requisite to their expansion. However studies to determine if these cells are able to migrate from the nerve ongoing. Finally we have initiated lineage tracing of the nerve origin of the osteoblasts through induction of HO in animals which possess a tamoxifen regulated Wnt1-Cre X R26R-YFP. Results suggest a substantial expansion of the cells within the nerves, but we did not observe YFP expression outside the nerve structure or within the mature bone. Interestingly, FACS analysis suggested that the Wnt1-YFP expression correlated with claudin 5, however a large percentage of the claudin 5⁺ cells during HO did not possess the YFP marker. Thus we hypothesize that Wnt1 may be on earlier stem cells, that repopulate the endoneurial progenitors, after they have expanded and migrated to undergo osteogenesis. Thus, the claudin 5⁺ cells are not Wnt1⁺ neural crest stem cells, but most likely a downstream progenitor. We propose to transfer the Wnt1-lineage marker YFP into these cells by delivery of tamoxifen to newborn mice and through pre-treatment with factors that induce neuroinflammation thus allowing the downstream progenitors to obtain the lineage tag. These experiments are ongoing and should be completed by the next reporting cycle. We are

also initiating cytokine bead array experiments to identify the key factors involved in the neuro-inflammatory response. This has been linked to M2 inflammatory pathways, involved in tissue remodeling, but to date no one has yet developed a signature. Thus we will not only include a variety of cytokines reported to be involved in neuroinflammation, but additional factors linked to M2 and M1 pathways. We will include M1 pro-inflammatory cytokines; because we will establish that these do not change, again demonstrating the link to M2 pathways. Again these studies are ongoing and will be reported on within the next period.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science*, *Military Medicine*, etc.).

- [1] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, *Science* 284 (1999) 143-147.
- [2] L. Armstrong, M. Lako, N. Buckley, T.R. Lappin, M.J. Murphy, J.A. Nolte, M. Pittenger, M. Stojkovic, Editorial: Our top 10 developments in stem cell biology over the last 30 years, *Stem Cells* 30 (2012) 2-9.
- [3] M. Pittenger, Sleuthing the source of regeneration by MSCs, *Cell Stem Cell* 5 (2009) 8-10.
- [4] M.N. Woszczyzna, A.A. Biswas, C.A. Cogswell, D.J. Goldhamer, Multipotent progenitors resident in the skeletal muscle interstitium exhibit robust BMP-dependent osteogenic activity and mediate heterotopic ossification, *J Bone Miner Res* 27 (2012) 1004-1017.
- [5] V.Y. Lounev, R. Ramachandran, M.N. Woszczyzna, M. Yamamoto, A.D. Maidment, E.M. Shore, D.L. Glaser, D.J. Goldhamer, F.S. Kaplan, Identification of progenitor cells that contribute to heterotopic skeletogenesis, *J Bone Joint Surg Am* 91 (2009) 652-663.
- [6] E. Salisbury, E. Rodenberg, C. Sonnet, J. Hipp, F.H. Gannon, T.J. Vadakkan, M.E. Dickinson, E.A. Olmsted-Davis, A.R. Davis, Sensory nerve induced inflammation contributes to heterotopic ossification, *J Cell Biochem* 112 (2011) 2748-2758.
- [7] N. Yosef, R.H. Xia, E.E. Ubogu, Development and characterization of a novel human in vitro blood-nerve barrier model using primary endoneurial endothelial cells, *J Neuropathol Exp Neurol* 69 (2010) 82-97.
- [8] T. Nitta, M. Hata, S. Gotoh, Y. Seo, H. Sasaki, N. Hashimoto, M. Furuse, S. Tsukita, Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice, *J Cell Biol* 161 (2003) 653-660.
- [9] A. Taddei, C. Giampietro, A. Conti, F. Orsenigo, F. Breviario, V. Pirazzoli, M. Potente, C. Daly, S. Dimmeler, E. Dejana, Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5, *Nat Cell Biol* 10 (2008) 923-934.
- [10] N. Takakura, H. Yoshida, Y. Ogura, H. Kataoka, S. Nishikawa, S. Nishikawa, PDGFR alpha expression during mouse embryogenesis: immunolocalization analyzed by whole-mount immunohistostaining using the monoclonal anti-mouse PDGFR alpha antibody APA5, *J Histochem Cytochem* 45 (1997) 883-893.
- [11] M.I. Garcia-Castro, C. Marcelle, M. Bronner-Fraser, Ectodermal Wnt function as a neural crest inducer, *Science* 297 (2002) 848-851.
- [12] J.L. Lewis, J. Bonner, M. Modrell, J.W. Ragland, R.T. Moon, R.I. Dorsky, D.W. Raible, Reiterated Wnt signaling during zebrafish neural crest development, *Development* 131 (2004) 1299-1308.
- [13] J. Duan, C. Gherghe, D. Liu, E. Hamlett, L. Srikantha, L. Rodgers, J.N. Regan, M. Rojas, M. Willis, A. Leask, M. Majesky, A. Deb, Wnt1/betacatenin injury response activates the epicardium and cardiac fibroblasts to promote cardiac repair, *EMBO J* 31 (2012) 429-442.
- [14] C.M. Gherghe, J. Duan, J. Gong, M. Rojas, N. Klauber-Demore, M. Majesky, A. Deb, Wnt1 is a proangiogenic molecule, enhances human endothelial progenitor function, and increases blood flow to ischemic limbs in a HGF-dependent manner, *FASEB J* 25 (2011) 1836-1843.
- [15] R.A. Deckelbaum, G. Holmes, Z. Zhao, C. Tong, C. Basilico, C.A. Loomis, Regulation of cranial morphogenesis and cell fate at the neural crest-mesoderm boundary by engrailed 1, *Development* 139 (2012) 1346-1358.
- [16] S.A. Kuznetsov, N. Cherman, P.G. Robey, In vivo bone formation by progeny of human embryonic stem cells, *Stem Cells Dev* 20 (2011) 269-287.
- [17] D. Medici, E.M. Shore, V.Y. Lounev, F.S. Kaplan, R. Kalluri, B.R. Olsen, Conversion of vascular endothelial cells into multipotent stem-like cells, *Nat Med* 16 (2010) 1400-1406.
- [18] E.A. Salisbury, Z.W. Lazard, E.E. Ubogu, A.R. Davis, E.A. Olmsted-Davis, Transient brown adipocyte-like cells derive from peripheral nerve progenitors in response to bone morphogenetic protein 2, *Stem Cells Transl Med* 1 (2012) 874-885.

APPENDICES: