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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. From these studies we have identified and isolated osterix ⁺ cells as a unique osteoprogenitor within the endoneurium of peripheral nerves. These cells express not only the osteogenic factors osterix and dlx5, but also the neural stem cell markers p75, musashi, and PDGFRalpha. Osterix ⁺ cells within the endoneurium are observed 24 hours after induction and then disappear. Simultaneous with there disappearance in the endoneurium, they reappear in the blood stream, and are now expressing claudin 5, a unique tight junction molecule that regulates the "blood-nerve barrier". These cells also now express the markers of extravasation, CD44 and CXCR4. These cells then disappear from the blood at approximately 3-4 days after induction, and simultaneously they are deposited at the site of bone formation. These cells appear to be associated with new vessels that have been rapidly synthesized at the site. This occurs prior to cartilage formation, suggesting that this pathway can be effectively targeted. Further we are currently working to develop this as a tentative blood diagnostic.					
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INTRODUCTION:

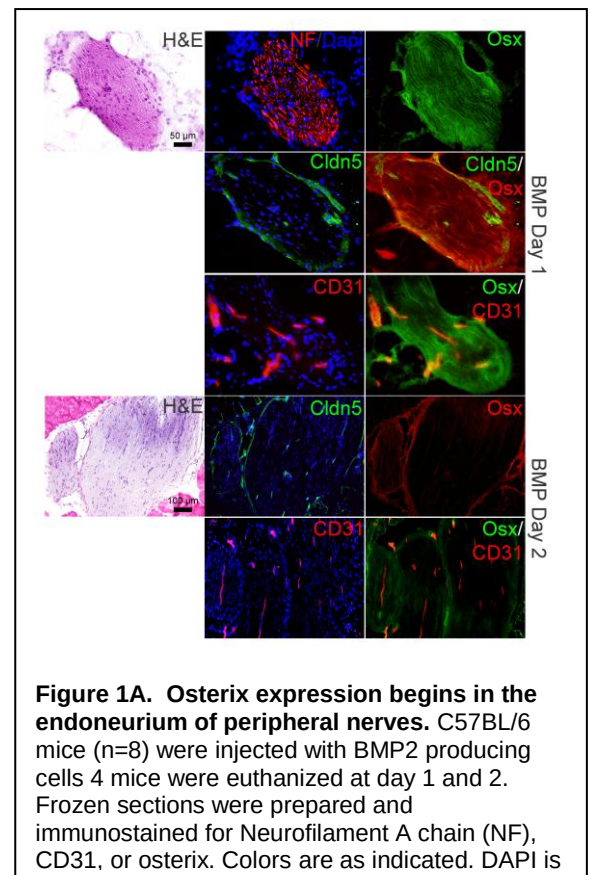
Heterotopic ossification (HO), the formation of bone in the muscle or other soft tissue, or any non-skeletal site can cause severe problems of pain and disability. It often requires the patient to undergo additional surgery. A particularly frustrating problem in amputees is the growth of bone within 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, have some efficacy in preventing HO in patients at high risk, but cannot be implemented in the majority of cases. Thus, there are currently no available efficacious treatments. Although the incidence of HO in the general populations 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. This process involves regulating the unique “blood-nerve barrier” to permit cells to exit from inside the endoneurial (axonal) compartment. This barrier is highly regulated and even proteins such as IgG are unable to enter. Intriguingly our data suggests a mechanism by which the cells exit through the vasculature, by expression of claudin 5, a unique tight junction molecule. We are currently characterizing the claudin 5 tight junction protein to determine if this factor is identical to the one shown to be necessary for the vascular regulation of this barrier, or a modification, that will provide the ability to open the barrier in the presence of these cells. It is intriguing that others have reported the presence of circulating proteins (claudin 5 one of them) in cases of trauma or concussive force, where the barrier has potentially been momentarily opened in humans. Thus we propose that the HO observed in military populations may be linked to the opening of this barrier, with these stem cells then entering the blood stream. During bone injury, they are recruited to the site of bone formation. Thus, there are many drugs that could currently intervene to block uptake of the cells. Therefore as noted in this report, we have filed a disclosure on these osteoprogenitors, and have described the potential to use them as a blood diagnostic for earlier intervention. Additionally, we have identified two other circulating factors that have been reported to be elevated in humans with severe HO, and are observed in our model as a component for opening of the barrier and release of these cells. We predict in the next 3-6 months we will have a cocktail of specific circulating factors that will provide a signature for HO and would potentially be set to start a clinical study to confirm our animal studies.

BODY:

Task 1: To isolate and characterize the nerve stem/progenitor population:

Previous studies suggested that a cell present in the endoneurial compartment of peripheral nerves express the osteogenic factor osterix after delivery of AdBMP2-transduced cells in the presence of cromolyn. Therefore, tissues were isolated and immunostained on days 1, 2, 4, and 7 after induction of HO through delivery of AdBMP2 transduced cells. Surprisingly, there was significant osterix expression on cells in the endoneurium at 24 hrs and even in the absence of cromolyn, but this rapidly disappeared within 24 hours as seen on tissues isolated 2 days after induction of HO (figure 1A). Note in figure 1A two different antibodies represented by two different secondary antibodies were used to confirm this phenomenon and patterns of osterix expression appeared to be similar, independent of the antibody. To confirm the nerve structure, tissues were immunostained with neurofilament H chain (NF). No osterix expression was observed on any subsequent days. Two cell types are common within the endoneurial compartment, specialized vascular endothelial cells and Schwann cells necessary for myelination of axons. Claudin 5 has previously been shown to be a marker for the specialized endoneurial endothelial cells. Therefore, tissues were co-stained for osterix and claudin 5 (figure 1A). Claudin 5 appeared to be associated with vessels, the expression pattern matching CD31⁺ vasculature shown on a serial section (figure 1A). Interestingly, the majority of the claudin 5⁺ cells no longer co-aligned with osterix. However by the second



day after induction, the cells were totally absent from the nerve. Examination of tissues isolated 4 and 7 days after BMP2 induction (Figure 1B) shows co-expression of osterix and claudin 5 in many cells throughout the muscle. Additionally, substantial vessel networks, as assessed by CD31 staining (red, Figure 1B), were observed in the region where the claudin 5⁺ osterix⁺ cells were localized, and these regions were found by day 7 to be associated with bone matrix (Figure 1B, yellow arrows). The data suggests that osteoprogenitors outside the nerve express claudin 5, however the osterix⁺ cells in the endoneurium 24 hours after delivery of BMP2 do not correlate with claudin 5.

The results show the presence of osterix⁺ cells in the endoneurium immediately after HO induction (Figure 1A). These cells then disappear at the same time as the appearance of osterix⁺ claudin 5⁺ cells in the circulation (Figure 2A). Within 4 days after induction of HO they disappear from the bloodstream simultaneously with an increase in expression of factors involved in extravasation and the appearance of the osterix⁺ claudin 5⁺ cells in muscle at the site of HO (Figure 3A). The data suggest that these osteoprogenitors exit the nerve through the BNB, enter the circulation, and home to the site of HO. In the first 24 hours after induction we observe both osterix and CD31 expression in individual cells but do not observe co-expression of osterix and CD31 in the same cell. This means that osteoprogenitors are not derived from endoneurial endothelial cells.

However, 48 hours after induction both CD31⁺ as well as osterix⁺ cells can be observed in endoneurial vessels, but it is not clear whether these two markers are expressed in the same cell at this later time.

We next immunostained sections for the presence of the Schwann cell marker and the major protein in peripheral myelin, myelin protein zero (MPZ) [6] as well as low-affinity nerve growth receptor (p75 NTR, Fig. 1C). Analysis by others has revealed expression of the pan neurotrophin receptor p75 (p75NTR) in neural stem cells [27]. It has been demonstrated that Schwann cells express elevated levels of p75 (NTR) during peripheral nerve regeneration and myelination because they too are derived from neural stem cells. However, it has also been shown that neural crest stem cells express p75 (NTR). To distinguish Schwann cells from neural progenitors, tissues isolated 1 and 6 days after induction of HO were co-immunostained with osterix and either p75 (NTR) or MPZ. Results obtained 1 day after induction show the coexpression of osterix and p75 (NTR) within the endoneurium of peripheral nerves (Fig. 1C, yellow regions in Day 1 merge as indicated by the yellow arrow). On Day 6 after induction, p75 is still strongly expressed in many cells in the area of bone formation. However, it is not clear if any of the cells expressing osterix (green, Fig. 1C) also express p75NTR (Fig. 1C) perhaps indicating downregulation of p75NTR during osteoprogenitor maturation. On the first day after induction, almost all nerves express MPZ in rings created by myelinating Schwann cells around each axon. However, cells expressing MPZ did not express osterix (Fig. 1C). This can be more easily seen in the

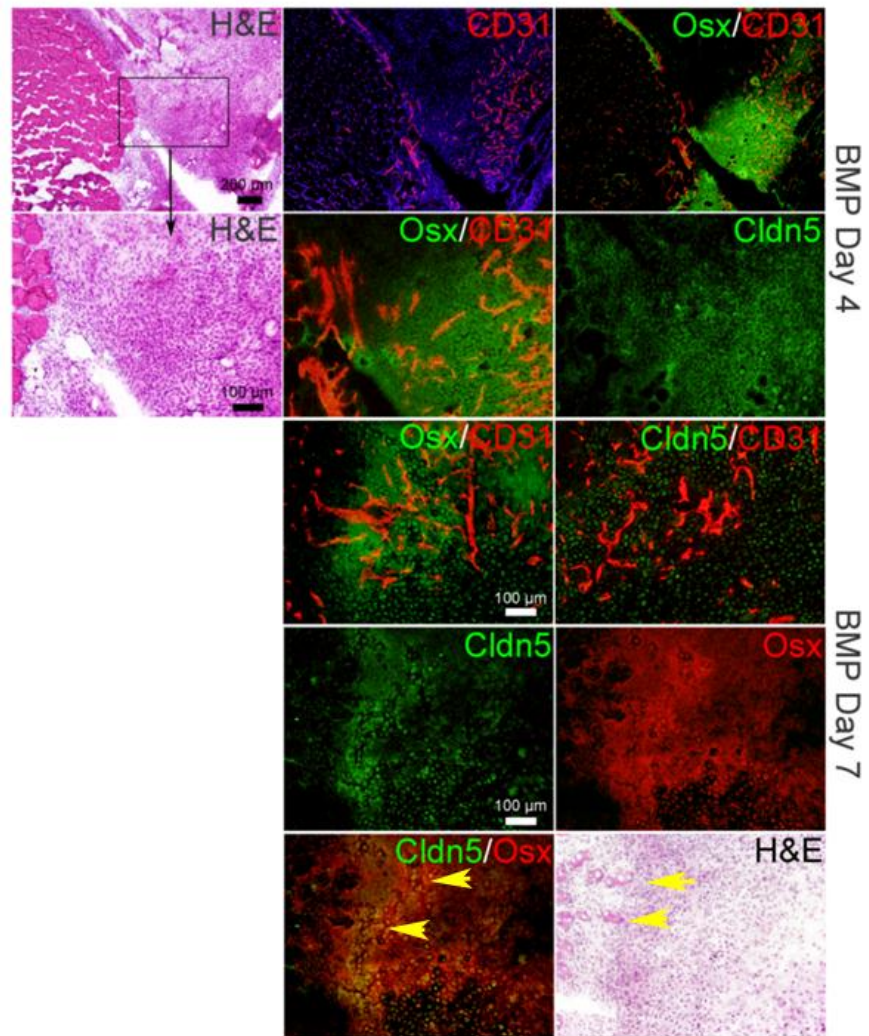


Figure 1B. After BMP2 induction the tight junctional molecule claudin 5 is expressed in vessels (CD31 positive), but later, after exit from the nerve, is expressed in cells outside of vessels. C57BL/6 mice (n=16) were injected with BMP2 producing cells 2 mice were euthanized at day 2 through 6. Frozen sections were prepared and immunostained for CD31 (red) or claudin 5 (green). DAPI is blue.

images taken at higher magnification in the second panel of Figure 1C. There are many areas where osterix⁺ MPZ⁺ cells are clearly present (white arrows, Fig. 1C). These areas are also reminiscent of published images of cells undergoing asymmetric DNA replication with groups of cells of varying sizes although this cannot be definitively discerned from these images. However, the results do indicate that osterix-expressing cells are not derived from myelinating Schwann cells. On the sixth day after BMP-2 induction, the peripheral nerves observed still express MPZ, but they did not express osterix (Fig. 1C). The results suggest that the osterix⁺ cell within the endoneurium is either a Schwann cell that is not myelinating or a neural progenitor that resides in the endoneurium of adult nerves as previously described by Morrison *et al.* Additionally, the results also strongly suggest that the nerves that contain osterix⁺ cells within the endoneurium also contain nonmyelinating Schwann cells, because we do not observe MPZ staining in certain discrete regions of these nerves (Fig. 1C). In total the result makes it quite likely that at least some of the osteoblasts used in HO are ultimately derived from either neural stem or nonmyelinating Schwann cells residing in adult peripheral nerves.

Osterix expression was present on the endoneurial cells for only 24 hours, suggesting that either expression is down regulated or that the cells immediately exit the nerve. One of the only ways for cells to exit the endoneurium is through the highly regulated tight junctions, which involves a degeneration of the junction resulting in the circulation of S100B and claudin 5 proteins. Since the osterix⁺ cells within in the tissues possessed claudin 5 (fig 2A) mononuclear cells were next isolated from the blood, sorted using FACS, and the claudin 5⁺ and ⁻ populations spun onto slides where we

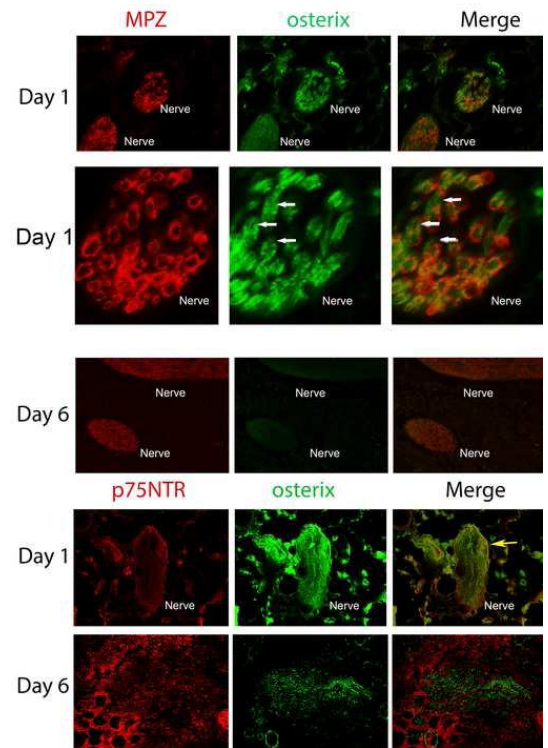


Figure 1C. Osteoprogenitors for HO are not derived from dedifferentiating Schwann cells.

Osteoprogenitors in peripheral nerves were assessed at early (1 day) and late (6 days) times after BMP-2 induction by analyzing frozen serial sections of C57BL/6 mice (n = 4 per group) euthanized either 1 or 6 days after BMP-2 induction. Sections were analyzed by immunohistochemistry simultaneously for either p75 (NTR) and osterix or MPZ and osterix. Colors are as indicated. The second panel of the osterix and MPZ stain on Day 1 is a higher magnification of the first panel where it can more easily be seen that cells that express osterix do not express MPZ.

Localized Delivery of Ad5BMP2 Transduced Cells Renders a Transient Presence of Claudin 5 Positive Cells in Circulation

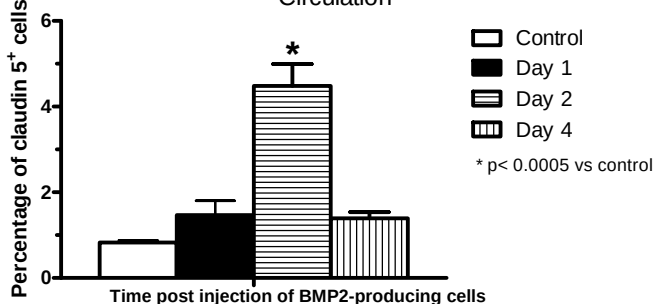


Figure 2A. Cells expressing claudin 5 increase in blood after BMP2 induction. C57/BL6 mice (n=4 per group) either remained untreated or were injected with BMP2-producing cells. Mice were bled by cardiac puncture at 0 (untreated), 1, 2, and 4 days after induction. Mononuclear cells were collected, reacted with an antibody to claudin 5 and subjected to FACS. Descriptive statistics was used to analyze the study results. The sample size in the groups was n=3. The analysis of variance (ANOVA) with Bonferroni-Holm post-hoc correction for multiple comparisons was used to detect statistically-significant differences between the number of claudin 5⁺ cells present in the circulation at given time points after intramuscular injection of BMP2-producing cells. The thresholds for statistically-significant differences were set at p<0.05.

immunostained for the presence of osterix. The results show that approximately 1 percent of the cells were positive for claudin 5 at 1 day after induction of bone formation however; there was no significant difference when compared to the control. Interestingly, this number rose dramatically at 2 days after the induction of bone formation, with approximately 4.5% of the cells now positive for this marker. However, the increase in cells expressing claudin 5 in blood was short-lived and levels had returned to background four days after BMP2 induction (Fig 2A).

To confirm that these circulating claudin 5⁺ cells were expressing osterix, both positive and negative populations were isolated by FACS followed by cytopsin and immunostaining for osterix. All of the osterix expression was found in the population of cells that were positive for claudin 5 (Figure 2B). The data collectively suggests that cells in the endoneurium that express osterix

rapidly exit the nerve, through vascular tight junctions that are regulated in part by claudin 5 and then circulate out of the nerve to the site of new bone formation. Upon entering the endoneurial vessels it appears that osteoprogenitors begin to express claudin 5, although the reason for such expression is unclear.

Others have reported the presence of circulating osteoprogenitors and many studies have been

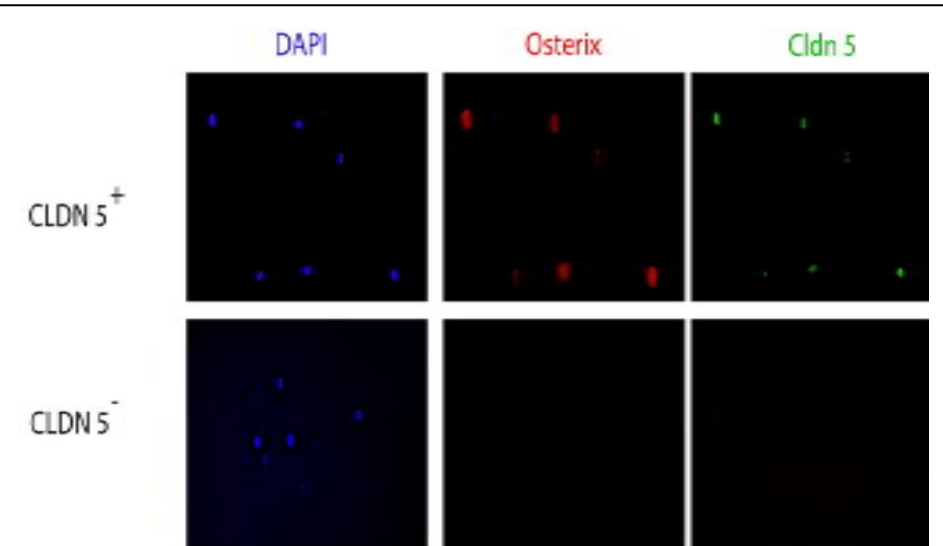


Figure 2B. Claudin 5⁺ circulating osteoprogenitors express osterix. Cells were isolated from muscle two days after BMP2 induction and claudin 5⁺ and ⁻ cells isolated by preparative FACS. Each population was then subjected to cytopspin and the resultant slides reacted with an antibody to osterix (red). Claudin 5, green; DAPI, blue. Claudin 5 positive population, A, B, and C; Claudin 5 negative population, E, F, and G).

conducted to define the mechanism of how these mesenchymal stem cells home and engraft in tissues. One process known as extravasation suggests that the circulating cells bind to their target through specific surface molecules. Through this binding process the cells can then pass through tiny pores in the vessels. To determine if this process was involved in the exiting of the claudin 5⁺ osterix⁺ cells and their coordinate appearance within the tissues at the site of bone formation, RNA was isolated from this region at daily intervals for 1-7 days, and known extravasation factors (CXCR4, CD44, SDF, and P and E-selectin) were quantified through qRT-PCR (figure 6D-F). The results show a significant increase in

CXCR4, CD44 and E-selectin RNAs starting 4 days after induction of HO and remained elevated throughout the remainder of bone formation (Fig 3A), whereas SDF and P-Selectin did not show a significant change (data not shown). To further confirm the protein expression, claudin 5⁺ and ⁻ cells were isolated from the tissues, and immunostained for these factors. As expected, based on studies in the literature, the positive population

appeared to also express CD44 and CXCR4 whereas the negative population expressed E-selectin (Fig 3B), suggesting that this process may be responsible for the engraftment of the cells at the site of bone formation.

Claudin 5-positive and -negative populations of cells were isolated by FACS from the tissues surrounding the site of new bone formation at day 4 (Fig 4) and cytopspin preparations immunostained for the osteogenic factors osterix and Dlx5 to assess co-localization with these markers. Surprisingly, the majority (90%) of the claudin 5-positive population also stained positively for osterix (Figure 4, panels A-C). Although there were numerous cells in the claudin 5⁻negative population, as determined by DAPI staining (panel F), there were virtually no cells staining positively for osterix. We next performed immunostaining to detect the expression of Dlx5 on the claudin 5⁺ and ⁻ cell populations. Dlx5 is an osteogenic factor

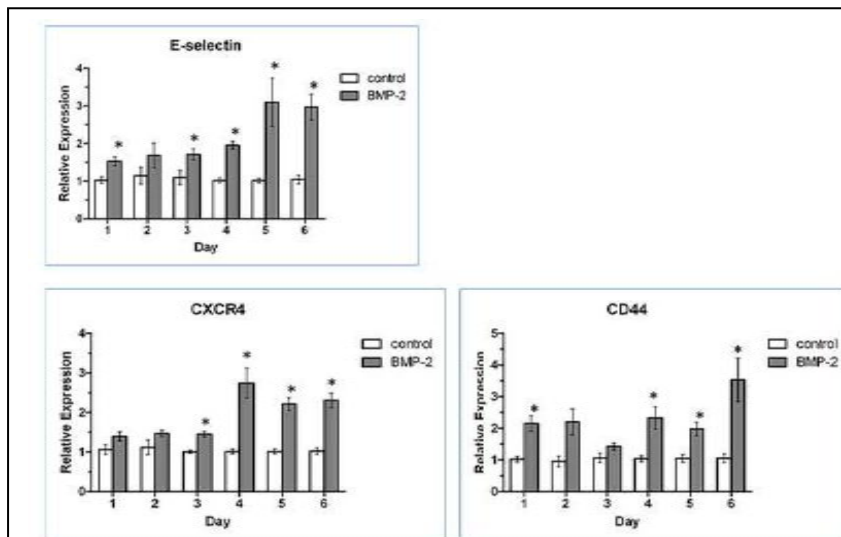


Figure 3A. CD44, CXCR4, and E-selectin are expressed upon BMP2 induction. C57/BL6 mice were injected with BMP2-producing cells (n=8 per group) and at the times indicated mice were euthanized, RNA extracted from muscle around the site of injection, and the relative amount of RNA encoding A) CD44; B) CXCR4, and C) E-selectin was determined and comparisons to determine statistically significant differences were made using an analysis of variance (ANOVA) with Bonferroni-Holm post-hoc correction for multiple comparisons. The thresholds for statistically-significant differences were set at p<0.05.

that is expressed during development in the perichondrial region of both the embryonic axial and appendicular skeleton (33) and is thought to be upstream of osterix. It is activated by BMP2 and upregulates both osterix

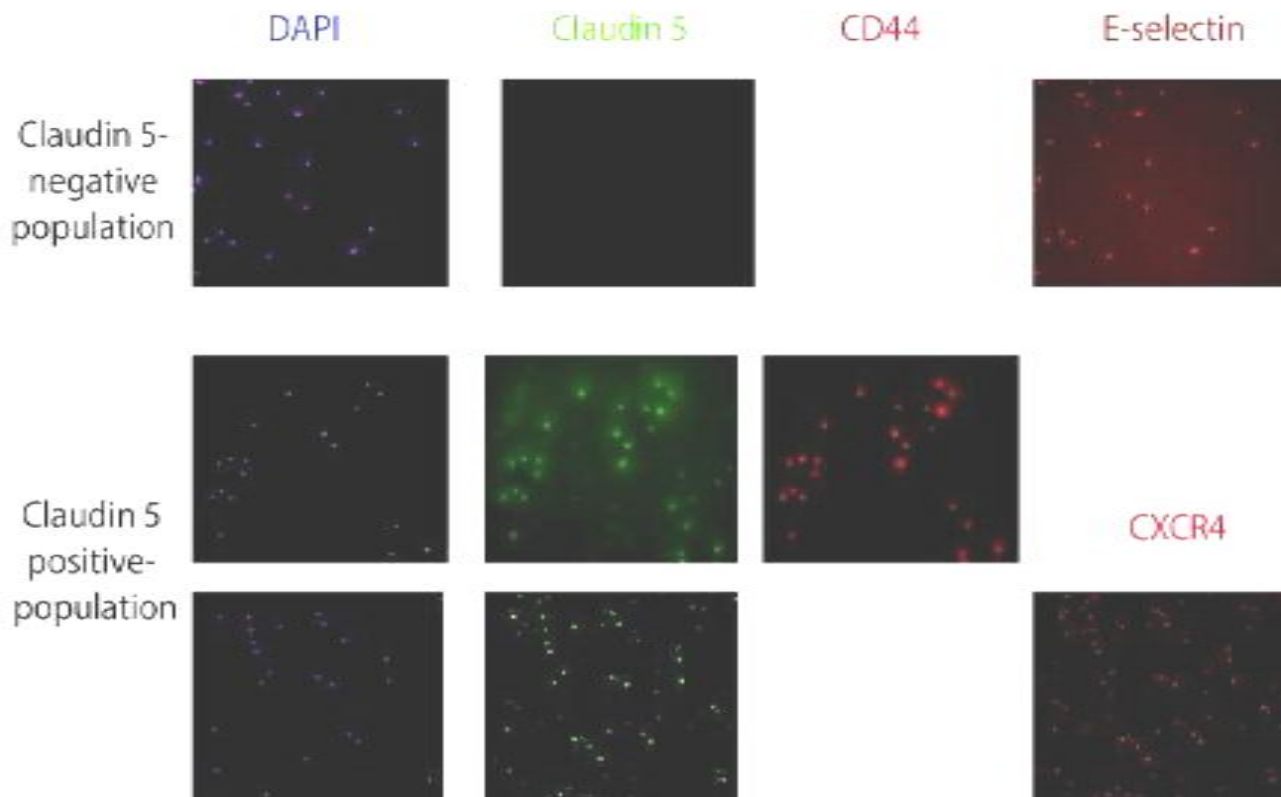


Figure 3 B. Claudin 5⁺ cells express CD44 and CXCR4, whereas claudin 5⁻ cells express E-selectin. C57BL/6 mice (n = 4 per group) were injected with BMP-2-producing cells and mice were euthanized 4 days later. Claudin 5⁺ and claudin 5⁻ cells were isolated by preparative FACS, subjected to cytopspin, and the resulting slides analyzed for staining for CD44, E-selectin, and CD44.

osteocalcin (30) expression during osteogenesis. Dlx 5 was expressed only in the claudin 5⁺ cell population, although some of the cells were not positive for this factor (Figure 4, panels G-I) the claudin 5⁻ population was completely negative for Dlx5 expression (Figure 4, panels J-L).

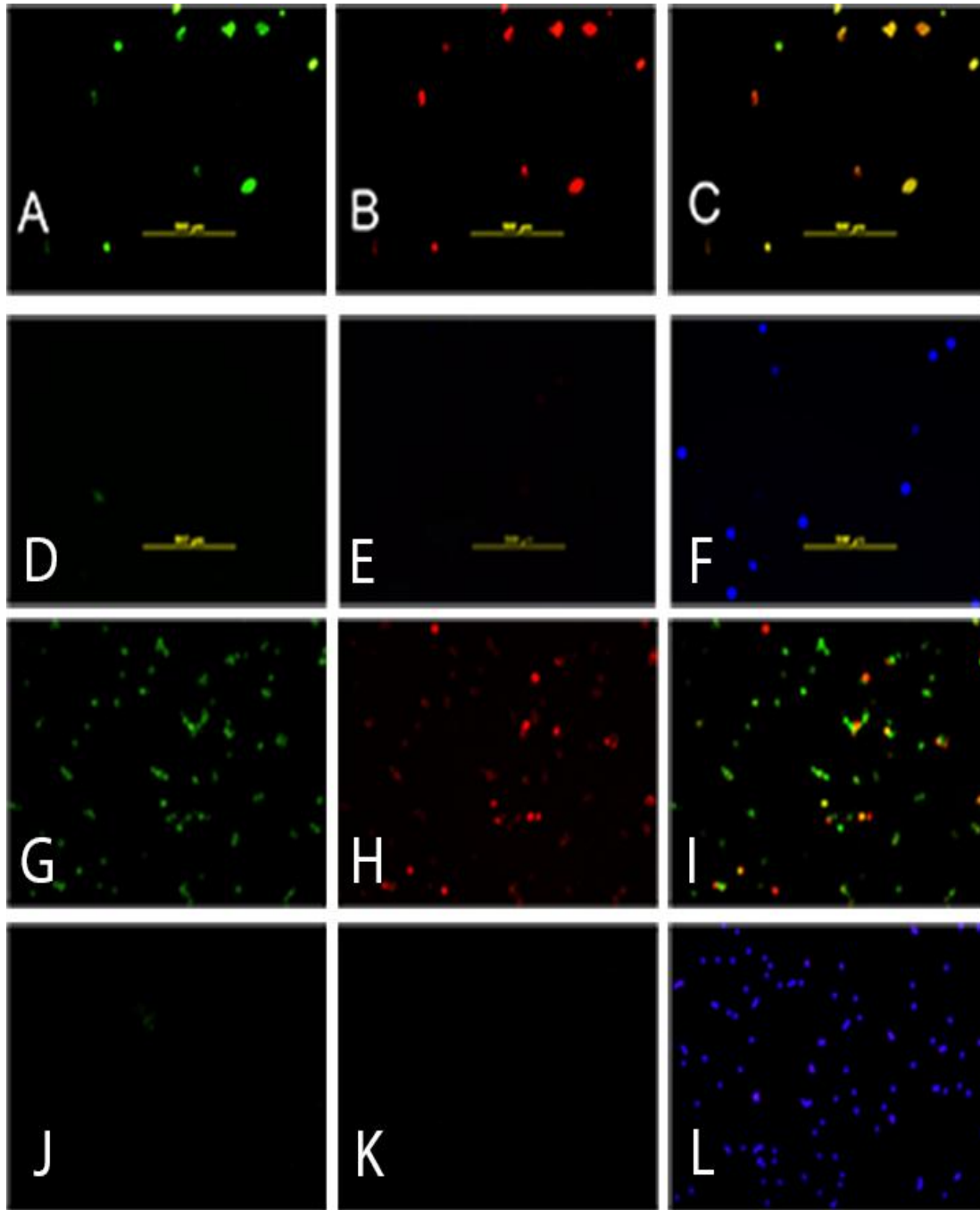
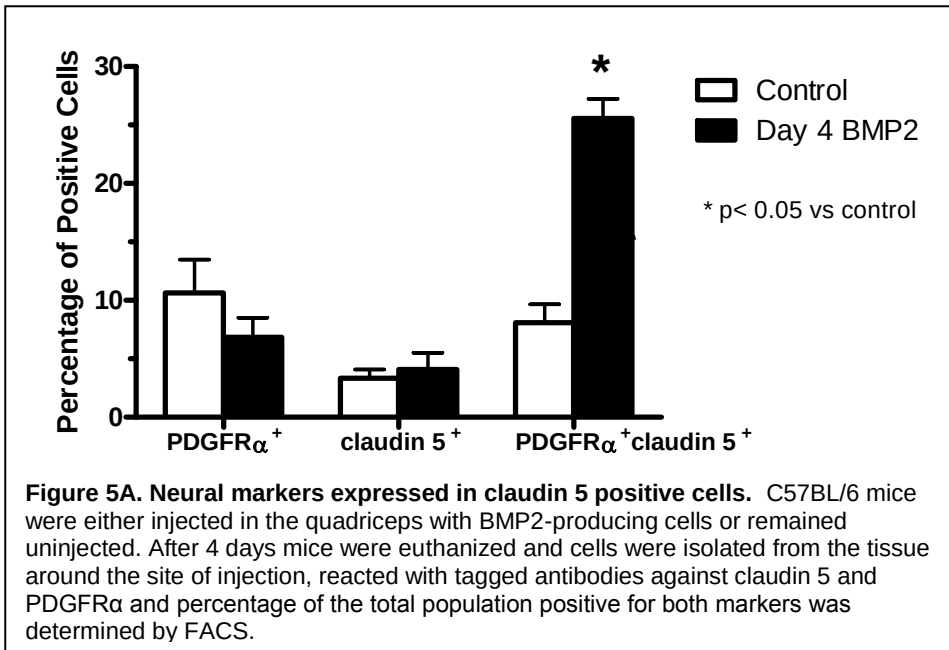


Figure 4. Panels A-L: Claudin 5⁺ cells express osteogenic markers. The claudin 5⁺ population (green) was isolated from a FACS of cells isolated from muscle 4 days after BMP-2 induction. These isolated cells were subjected to cytopspin and the slides were then probed with antibodies for claudin 5 (green) and osterix (red). **(A-C)** One field obtained from the claudin 5⁺ population with C being the merger of A and B; **(D-F)** one field of the cytopspin of a claudin 5⁺ population obtained from the same mouse that was stained with antibodies against claudin 5 (green) and osterix (red) as well as DAPI **(F)**. In the claudin 5⁺ cell population, osterix-positive cells were found to be 75% ± 3%. **(G-I and J-L, respectively)** the cytopspin patterns of the claudin 5-positive and negative populations of another mouse after staining for claudin 5 (green) and *dlx 5* (red).

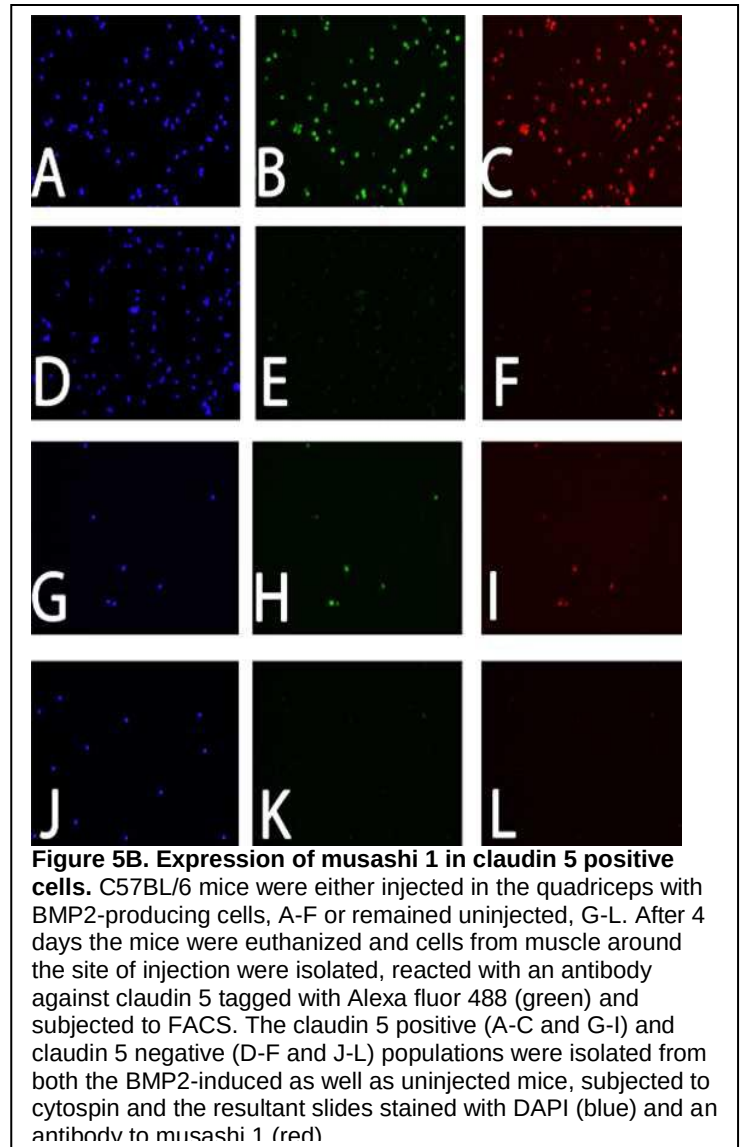
We next looked at the expression of two additional factors characteristic of nerve stem/progenitor cells, PDGFR α and musashi. Both PDGFR α , a factor associated with perivascular astrocytes and shown to be involved in glial-endothelial cell interactions and critical for maintenance of the blood-brain barrier, and musashi, which is an RNA binding protein that is highly specific for neural stem cells and which is not expressed in fully differentiated cells, were assessed on the claudin 5⁺ cells. FACS analysis of the claudin 5⁺ cells showed that PDGFR α was expressed on these cells (Figure 5A). Although we did not observe



a claudin 5⁺ PDGFR α ⁺ population we did observe a small PDGFR α ⁺ claudin 5⁻ population (Figure 5A) that may represent Schwann cells, which previously have been shown to be positive for this receptor. Interestingly, this marker has also been shown to be on osteoblast progenitors isolated from military patients with early HO.

We next FACS-selected the claudin 5⁺ and ⁻ populations from the cells isolated from tissues two days after delivery of the AdBMP2-transduced cells centrifuged them onto slides, and immunostained for musashi. Almost all the cells in the claudin 5-positive population were also positive for musashi (Figure 5B, panels G-I). Positive immunostaining for musashi was not observed in the claudin 5-negative population although there were an equal number of cells on the slides (Figure 5B, panels J-L). Overall the data suggests that these osteoprogenitor cells, in addition to the upregulation of osteogenic factors, also express neural crest markers, which may be remnants of their neural origin.

Since others have reported the presence of PDGFR α on the surface of an osteoprogenitor expressing Tie 2, the claudin 5⁺ and ⁻ populations were immunostained for Tie-2 (Figure 6A). Almost all of the claudin 5⁺ cells were found to express Tie-2, but there appeared to be a wide variation in its expression level (Figure 6A). Additionally, we noted that in some cases Tie 2 had a surprisingly asymmetric localization on cells (Figure 6B), which may indicate a migrating, rather than matrix bound, cell as described by Saharinen *et al* (35). The data collectively suggest that these osteoprogenitors express not only osteogenic transcription factors (osterix and dlx 5), but also markers of early neural (musashi and PDGFR α), and vascular (Tie-2) progenitors.



Hence the progenitors, although bound for a potentially unique destination (bone), are expressing a variety of markers that may allow them to be recruited for a variety of fates.

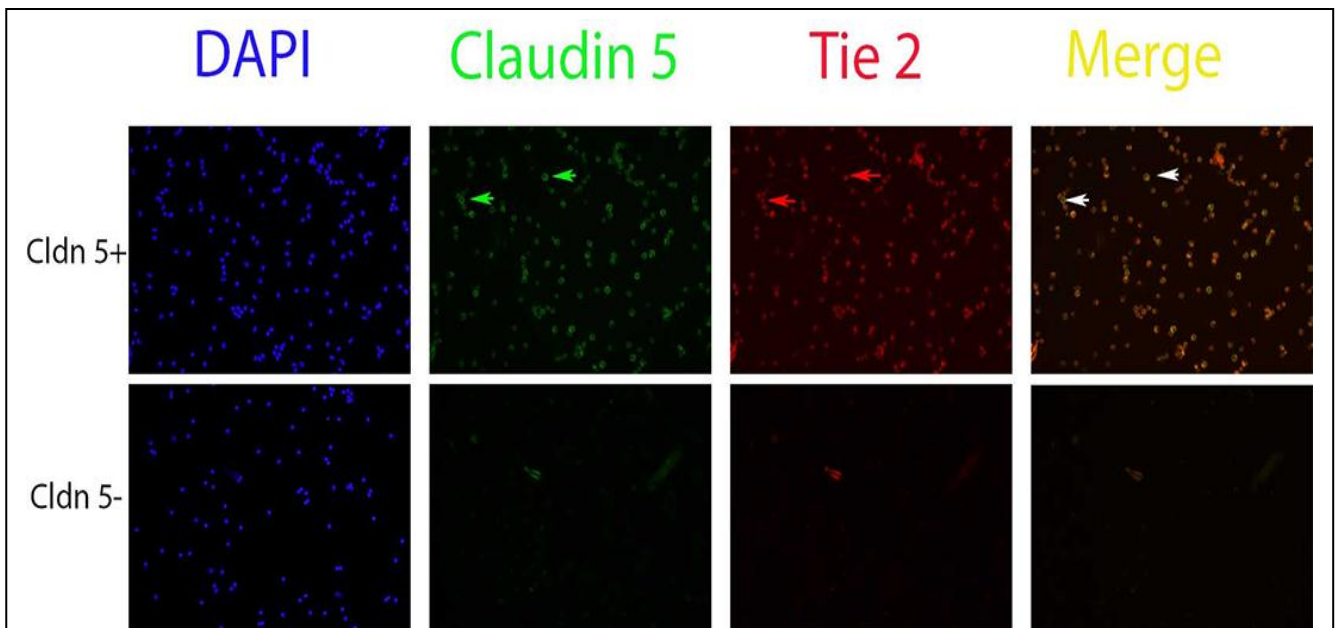


Figure 6 A. Osteoprogenitors express the endothelial marker Tie 2. C57/BL6 mice were injected with BMP2-producing cells (n=4) and four days after induction the mice were euthanized and cells harvested from the muscle around the site of injection were separated by FACS into claudin 5 positive and negative populations. The populations were subjected to cytopspin and the slides were assessed for expression of Tie2 (red). Claudin 5, green; DAPI, blue.

The data collectively suggests that the osterix⁺ endoneurial cell that appears within 24 hours after induction of HO possess characteristics of a neural crest stem cell observed migrating in the embryo to form the bones of the head and neck. It is intriguing that the data matches data found by others using a different system for inducing HO in mice, but here we have placed significantly more markers on these cells. Further previous reports suggest the cell was derived from the local vasculature, however, from these studies the cell appears to be derived from the nerve, but extravasate through the newly formed vessels, long before cartilage. Thus it is easy to see how one would think that the cell was being derived locally rather than systemically. Additionally the presence of PDGFR α is intriguing because of the work in human HO, which suggests the osteoprogenitor possesses this as one of the surface markers. Further, these cells possess all the markers reported on the human cells, again suggesting that this population may also be contributing to the human HO.

We have previously demonstrated that these cells also express markers of pluripotent stem cells, klf-4, nanog, Oct 4. Intriguingly, a colleague of ours recently demonstrated that enforced expression of these factors in adult cells was insufficient to drive them to pluripotent stem cells (iPS cells). His data suggested that activation of TLR3 was required for this to occur. Interestingly, the adult cells within the endoneurium appear to express these primitive (iPS) factors, but also express TLR3 suggesting that these osteoprogenitors may be formed from a program that can generate these iPS-like cells *in vivo*. Recent reports in the literature suggest that Schwann cell precursors within the endoneurium are capable of undergoing neurosphere development and functioning as neural stem cells *in vitro*.

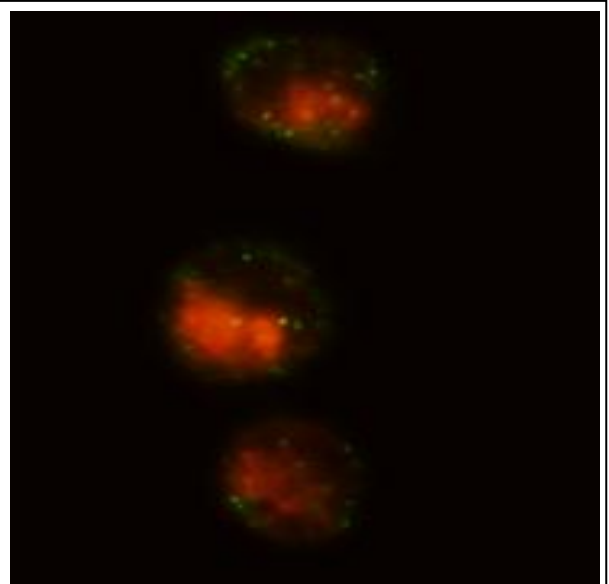


Figure 6 B. Osteoprogenitors express the endothelial marker Tie 2. C57/BL6 mice were injected with BMP2-producing cells (n=4) and four days after induction the mice were euthanized and cells harvested from the muscle around the site of injection were separated by FACS into claudin 5 positive and negative populations. The populations were subjected to cytopspin and the slides were assessed for expression of Tie2 (red). This is a representative photomicrograph of a 40X magnification showing an asymmetric distribution of Tie-2 (red) in some of the cells.

Therefore in this upcoming first quarter of this year we will attempt to confirm that these osterix⁺ endoneurial cells are capable of forming neurospheres (functioning as a neural stem cell) in vitro. Towards completing this, we isolated peripheral nerves from animals after induction of HO with AdBMP2, Adempty cassette transduced cells, or PBS (n=4 per group) and then dissociated the cells. The dissociated cells were plated at high density in neurosphere media, and allowed to form neurospheres for 3 weeks. At this point all samples formed tentative neurospheres but we noted that there were significantly more in the sample isolated after delivery of BMP2. The neurospheres were then dissociated and placed in media that would drive a neuron phenotype, the true test as to whether the tentative neurosphere contains a pluripotent cell. These dissociated cells did not have the same distinctive neuron-like characteristics in the two controls to suggest there were true neural crest stem cells; however, in the BMP2 dish there were handfuls of cells not seen in the other groups that appeared neuron like. We are currently characterizing these using immunocytochemistry to determine if they are expressing the correct markers. In the next studies we will isolate the osterix⁺ cells specifically by using mice possessing an osterix-cherry reporter. These cells will then be tested and compared to the other to determine if they are behaving similarly to iPSCs cells. If these cells appear positive in this assay, we will then subject them to a chick assay in which we generate a chimeric embryo. Understanding this basic information about these cells will be necessary to understanding further, what drives their generation, and how this process can be disrupted or prevented, particularly in cases of amputation, where the bone often just keeps coming back.

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 are attempting to employ two strategies. The first is lineage tracing which would selectively label the cells with a nerve specific marker (wnt-1) and then determine if the marker appears in the osteoblasts. We demonstrated in the previous report that using a tamoxifen regulated cre-recombinase animal that was crossed to a R26RFYP reporter (Ert-Wnt-YFP) that in the presence of tamoxifen, the tentative osterix⁺ were observed possessing the marker. However, when we attempted to see the marker in other cells not associated with the nerve it was variable, with several samples showing no reporter fluorescence, suggesting perhaps some technical problems.

To circumvent this we tested whether a different fluorescent reporter would provide more stability in analyzing the signal. Therefore we switch to a tomato red reporter, which we have now shown to be significantly more stable. Figure 7 shows a comparison between the YFP signal and the tomato red in these animals. As can be seen there are a handful of positive cells with the YFP whereas in the tomato red, using the same ERT-WNT1-cre animal to direct specificity, now we see a large number of cells positive for the reporter. This suggests that the potential YFP signal is being lost during the process of generating the tissue sections. **We are currently analyzing these mice to determine if we observe the tomato red in the osteoblasts associated with bone.**

In addition to these experiments we have also been attempting to selectively delete the osterix⁺ cells specifically from the nerve at the time of bone formation, to determine if this would block the appearance of these cells at the site of bone formation and overall bone formation. To do this, we obtained mice from Dr. Benoit de Crombrughe, which have a floxed osterix that would allow us through our ERT-WNT1-cre mouse to selectively delete osterix from the nerve population in an attempt to block bone formation. To obtain a fully deleted animal we next bred the mice through many crosses to obtain offspring that had two floxed alleles and the ERT-WNT1-cre element. We next established bone formation, and found that we did obtain bone. However, when analyzed the tissues we found that osterix did not appear to be floxed in the nerve. In discussions with Dr. de Crombrughe, it appears that it is quite difficult to obtain cre recombinase levels capable of floxing both alleles from our reporter. The suggestion was to use a deleted allele obtained from their knock out animal, and cross that into these mice, to obtain final offspring that possess ERT-WNT1-cre x floxed osterix allele 1, and deleted osterix allele 2. **We are currently breeding these. These experiments should be completed in the next 6 months.**

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. However, we are now also working on completing these experiments to determine if we can see the fluorescent marker in the wild type bone. To this end, we have generated WNT1-cre mice that are not tamoxifen regulated that possess a tomato red reporter, and a portion of the nerve will then be ligated into a

wild type mouse. Heterotopic bone formation will be established and we will then determine if the tomato red reporter is in the new osteoblasts. These experiments should be completed in the next 6 months. We are currently breeding the mice with the WNT1-cre to obtain enough offspring for these experiments.

Finally we will also isolate osteoprogenitors as described above, from an animal possessing a fluorescent reporter in all cells, and then inject them into wild type mice during bone formation. This will be done in two ways; the first is by isolating the progenitors from the blood, and injecting them, the second from the nerve itself. To isolate the cells from the nerve, we will need to breed the osterix-cherry into the GFP mice, so that we can harvest osterix⁺ cells by fluorescent cell sorting from the nerve that will also possess a GFP. In all cases the isolated cells will be injected into a recipient mouse that has either had HO induced through delivery of our AdBMP2-, Adempty cassette- transduced cells or PBS so that we can evaluate their potential to form bone. ***These experiments should be completed within the next year.***

Task 3: Identification of key changes in the blood profiles that may represent a potential biomarker for HO.

In this aim we proposed to take a global strategy to identify markers in the blood that could be beneficial for determining one's risk after traumatic injury of HO and could potentially localize the timing of when to provide therapeutic intervention. However, as aims 1 and 2 progressed, we identified several factors that would potentially allow us to take a focused approach at developing a diagnostic.

As described in aim 1, we identified that the osteoprogenitors circulate from the nerve and can be detected in the blood stream. These progenitors are expressing the unique marker claudin 5. We have recently filed a disclosure through BCM to enable us to use this marker as a blood diagnostic. Further, we are currently looking for a partner who can provide us patient blood for testing whether this phenomenon holds true in humans as well. Although the work is focused towards a military population, for feasibility we will obtain under an approved protocol human blood from patients after hip surgery that would potentially be given HO inhibitors that may not necessarily block the formation and release of these cells. We will seek DOD approval in the next month.

Additionally, previous work in the literature has demonstrated that often traumatic force that momentarily opens the blood nerve barrier such as blast injury is accompanied by elevated circulating levels of S100B, so we will focus on also measuring the levels of S100B in the patient blood.

Finally, based on the literature one of the only ways to open the blood nerve barrier is through activated MMP9 protein. Interestingly, studies in large animals including rat, show that BMP2 induces bone formation through a different type of mechanism that requires recruitment of cells from the periosteum. Thus it would be essential in using the recombinant protein to disrupt the periosteum and provide a source of progenitors, such as the current state of the art for spine fusion using recombinant BMP2. In studies in the other animal systems, we noted that the brown adipose does not produce active MMP9. Further there appears to be significant pro-MMP9 produced but it does not get cleaved to become active. As a result of the lack of MMP9, there is a build-up of these tentative osteoprogenitors within peripheral nerves adjacent to the site of heterotopic bone. We are currently focused through another award on characterizing and circumventing this problem. However here, we note that MMP9 activation is through cleavage by plasmin. Plasmin is produced by the liver, and found in the circulation as plasminogen the inactive form. In order for plasminogen to be cleaved to its active form plasmin, it must bind to platelets, which then hold the cleavage site in the right conformation to be able to be cleaved by either urokinase or tissue specific plasminogen activator. Interestingly, in rats, we observed high levels of urokinase, but we did not observe either plasmin or plasminogen, suggesting that this factor is not being recruited and activated. Therefore the lack of plasmin most likely results in the lack of MMP9 and inability to open the barrier and recruit the osteoprogenitors. However, personal communication with Drs. Jonathan Forsberg and Thomas Davis, suggests that military patients with problematic HO, appear to have difficulty clotting in the regions where HO is present. Since plasmin can also cleave clots, and induces fibrinolysis the patients may be clotting, but it is being degraded by the higher levels of plasmin due to the mechanism of HO. Interestingly, in patient samples from earlier HO in military personnel, all the MMP9 expression appears to be active similar to the mouse, but not the larger animals, suggesting that humans maybe utilizing this pathway. Further, in humans platelet rich plasma (PRP) may not be very effective, yet in animal studies, several suggest that it can enhance BMP2 induced bone repair. This mechanism would potentially explain the variability of PRP, which would place plasminogen in the tissues in animals lacking, which then can be rapidly cleaved and activated to generate active MMP9. Our collaborators are currently going back through their data to confirm the elevation in plasmin, and to look at a few other factors that are known to potentially circulate that are associated with this pathway.

We hope within the next year to create a signature of circulating factors that govern these processes, this will include these initial factors necessary for opening the barrier, the factors associated with the circulating

cells, and two specific adipokines that may be highly involved in regulating the osteogenesis. We plan to disclose these findings through a joint disclosure with the Navy and Drs. Forsberg and Davis. Additionally, we will complete our analysis of these factors in the circulation of mouse and rats, since they appear to use very different mechanism to drive bone formation. However, claudin 5 is unique enough that this single factor could potentially be developed as a diagnostic, so we will continue to also move forward with characterizing this factor in blood as well.

KEY RESEARCH ACCOMPLISHMENTS:

- Approval of animal experiments
- Characterized the osterix⁺ cells within the endoneurium. We have identified key markers on this cell including PDGFR α , claudin 5, musashi, CD44, CXCR4, dlx5, and tie2.
- Demonstration that the osterix⁺ cells also expresses the unique tight junction molecule claudin 5 necessary for exiting the blood nerve barrier to enter the circulation.
- Demonstrated the presence of claudin 5⁺ osteoprogenitor cells within peripheral blood prior to deposition at the site of heterotopic bone formation. **Therefore we have filed a disclosure describing the cells, and their usefulness as a blood diagnostic for HO.**
- Demonstrated that suppression of nerve remodeling through delivery of cromolyn, did not suppress the expansion of the claudin 5⁺ cells; but it did suppress their ability to exit the nerve.
- Preliminary data to demonstrate whether these tentative osteoprogenitors within the nerve are able to generate neurospheres, and eventually neurons. These experiments are ongoing, however, we noted the BMP2 had significantly elevated number of neurospheres form, and all cultures had more glial cell looking cells, however, the BMP2 had several neuron looking cells suggesting these may be pluripotent.
- We currently have three different approaches underway to confirm the functionality of these cells. The first is to perform lineage tracing with the Ert-Wnt-Cre:R26R tomato red mice, which should provide a method for following wnt1 progenitors in the nerve to see if they end up in bone. Secondly, we will isolate from the nerve these osteoprogenitors possessing a reporter, and then place them in to a wild type mouse and determine whether they incorporate into the structures of bone and cartilage. Third, we are breeding our floxed osterix allele into a heterozygous osterix^{-/-} mice to have a viable mouse with only one floxed allele, which will then be crossed to the Ert-Wnt1 cre to determine if that will selectively ablate the osteogenesis of these tentative progenitors during HO.
- We have identified a key pathway involved in opening this blood nerve barrier. In this pathway, MMP9 must be activated through cleavage by plasmin. In the absence of cleavage by plasmin, the proMMP9 remains associated with the transient brown adipose, and the osterix⁺ cells within the nerve continue to build up but are unable to be released. We have noted that in larger animals, the transient brown adipose appears to lack plasmin and plasminogen, which we propose is responsible for the build up of progenitors in the peripheral nerves surrounding the site of new bone formation. It is unclear why the larger animals do not appear to have either plasmin or plasminogen within the brown adipose as does the mouse and human, but we do note that it leads to the inability to activate MMP9, and the unique barrier. IN this absence the cells build up on the nerve, but do not result in heterotopic ossification. **Thus, this suggests a very POWERFUL and NOVEL therapeutic target for the prevention and treatment of HO.**

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

- Lazard ZW, Olmsted-Davis EA, Salisbury EA, Gugala Z, Beal II E, Ubogu EH, Davis, AR (2015) Neural Origin of Osteoblasts during Heterotopic Ossification. *Clinic Orthop and Related Res* (In Press).
- Disclosure: "Neural Derived Osteoprogenitors" inventors: Davis EA, Davis AR, Lazard ZW, Salibury EA, Gugala Z BCM technologies.
- Olmsted-Davis E. A., Salisbury E. A., Lazard Z.W., Gugala Z. and Davis A.R. Peripheral nerves are a significant reservoir of progenitor cells for bone formation in heterotopic ossification. *Bones & Teeth Gordon Research Conference*, Jan 26-30, 2014 Galveston, TX. (1st place poster prize)
- Olmsted-Davis EA, Sonnet C, Davis EL, Salisbury EA, Lazard ZW, Beal II E, Forsberg J, Davis T, and Davis AR. Heterotopic Bone is derived in large part from progenitors in local peripheral nerves: major

difficulties in large animal models for BMP2 testing. Musculoskeletal Biology and Bioengineering Gordon Research Conference (oral presentation) Aug 3-8, Andover, NH, USA

- Lazard ZW, Olmsted-Davis EA, Salisbury EA, Beal II E, Davis, AR Neural Origin of Osteoblasts during Heterotopic Ossification. Center for Cell and Gene Therapy, Nov 13-14, 2014 Galveston, TX.

CONCLUSION: We have identified a cell population in the endoneurium of peripheral nerves that expand during heterotopic ossification (HO). This cell appears maybe a pluripotent stem cell that expands within the endoneurium of peripheral nerves. Within 24 hours it is expressing the osteoblast specific factor osterix and dlx 5, but then these cells rapidly disappear from the nerve. They are then observed in the circulation at 48 hours, until about 4 days after induction of HO, when they now appear at the site of bone formation. The cells in the circulation are expressing osterix and dlx5, but now they also express the unique tight junction marker claudin 5, which allows them to be readily purified. This unique marker has been previously implicated to circulate in the blood following traumatic injury such as head trauma, where the blood nerve barrier may have been temporarily disrupted. We are currently working on obtaining human blood to characterize whether this could be a potential diagnostic for HO. Further, these cells express PDGFR α , CD44, and a number of additional markers that have been shown to be present on human osteoprogenitors, suggesting a common mechanism. We also have shown that these cells express primitive markers, klf4, nanog, musachi, and oct4, suggesting that these cells may have become pluripotent similar to an iPSC cell. These experiments are ongoing, and will allow us to then determine if the risk for HO is associated with activation of toll-like receptor 3, since activation of this factor is essential for iPSC transformation of adult cells, and if so the relationship to the types of wounds associated with HO, i.e. blast injury that could temporarily open and release these cells, to contaminated wounds, that could activate TLR3 and lead to a generation of the pluripotent progenitors, to BMP2 released from fracture that could drive an osteogenic phenotype. We are currently on schedule, and will answer these additional questions within the next year,