Abstract

Craniosynostosis is a common deformity involving premature fusion of the skull bones. A better understanding of the process by which the skull bones form (intramembranous ossification) could result in improved treatment options. Some skull bones are produced by a population of migrating, multipotent cells from the developing brain, known as neural crest cells (NCCs). In turtles, the bony plates that comprise the ventral part of the shell (the plastron) are also formed by intramembranous ossification. I am investigating whether similar migrating NCCs, arising from the developing spinal cord instead of the brain, form the plastron. Turtle NCCs were isolated, allowed to differentiate, and the resulting cell types were analyzed. The fraction that produced typical NCC-derived cells, such as pigment cells, was compared to the fraction that produced bone-forming cells. This experiment tested the model that NCCs migrating through the body of turtle embryos are capable of differentiating into bone.

Multipotency of Trunk Neural Crest Cells in *Trachemys scripta*

Craniosynostosis, which is characterized by the premature fusion of the intramembranous bones of the cranium at one or more sutures, is a relatively common developmental disorder, affecting 1 in 2000-2500 children (Boulet *et al*., 2008). The initiation and progression of intramembranous ossification – the growth of flat bones like the skull bones – remains poorly understood, while endochondral bone formation – the process by which long bones form using a cartilage template – is well described. If the processes controlling intramembranous osteogenesis were better understood, improved treatment of craniosynostosis might be possible.

Background

Even though intramembranous ossification is not entirely understood, the contribution of neural crest cells to the forming bone has been established as a key feature. Neural crest cells are a population of multipotent cells that migrate from the folds of the neural tube, a structure which ultimately becomes the organism's brain and spinal cord. This population is divided into groups based on the region of the neural tube from whence they originate, which also influences the types of cells that they can differentiate into (Gilbert, 2014). Trunk NCCs arise from most of the spinal cord from the neck down and are capable of producing neurons, glial cells, and melanocytes. Cranial NCCs arise from the midbrain and hindbrain and form many structures in the head. Cranial NCCs can differentiate into melanocytes, glial cells, and peripheral neurons, just as trunk NCCs can, but they are also able to form bone, cartilage, and connective tissue (Gilbert, 2014). The bones of the face and the anterior portion of the skull are derived from cranial NCCs (Le Lièvre, 1978). In mammals, the division between mesoderm-derived bone and NCC-derived bone lies along the coronal suture joining the frontal and parietal bones (Jiang *et al*., 2002; Yoshida *et al*., 2008).

The turtle plastron may provide a more tractable model to study intramembranous ossification than the skull. The plastron is a ventral plate composed of nine bones that form by intramembranous ossification with sutures connecting the plates allowing a direct comparison with skull bones (Gilbert *et al*., 2001). As discussed above, cranial NCCs are a major contributor

to the skull bones, and Cebra-Thomas *et al*. (2007) indicated that *trunk* NCCs play a role in the formation of the plastron. After the initial wave of NCCs (equivalent to those found in other vertebrates) has concluded its migration, a second set of NCCs migrate away from the neural tube. These trunk NCCs express *PDGFRα* (also expressed in bone-forming cranial NCCs, but not typical trunk NCCs), and other typical NCC markers (Cebra-Thomas *et al*., 2013). This array of expression in these late-migrating trunk NCCs suggests that they may be capable of osteogenesis. Thus, it has been proposed that the plastron bones are derived from this turtle-specific second wave of trunk NCCs (Cebra-Thomas *et al*., 2013; Rice *et al*., 2016).

Methods

To support this proposal, I attempted to analyze the relative abundance of the different cell types that arose from the second waves of turtle trunk NCC migration. To accomplish this, I chose antibodies that bind to specific cellular proteins. After allowing these primary antibodies to attach to the proteins, I labeled the primary antibodies with another set of antibodies (secondary antibodies). The secondary antibodies contain a fluorescent tag that enabled me to see where they are (and, by extension, where the specific proteins are) with a fluorescent microscope. I used primary antibodies against proteins expressed by neurons and osteoblasts (in addition to a stain to locate nuclei).

In July 2016, trunk NCCs from the second migration were isolated and allowed to differentiate for about two weeks. After they were fixed in preparation for staining, an antibody against neurons (anti-neurofilament) and another against bone (anti-osteopontin) were used simultaneously and then marked with different secondary antibodies. Each secondary antibody was conjugated to a different color fluorochrome, allowing the both staining patterns to be examined individually. Hoescht nuclear stain was applied at this point to make the nuclei fluoresce blue. The stained cells were mounted on slides and photographed using a fluorescent compound microscope to examine each fluorochrome.

Results

As described above, staining simultaneously with two or more antibodies maximizes the information that can be collected from each group of cells. This procedure yielded the first results, which came from a small-scale, preliminary experiment comparing different antibodies that stain bone-forming cells (osteoblasts). These results were promising, showing positive staining for bone proteins but no staining for neuron proteins (Fig. 1). This suggested that differentiated late-migrating NCCs contain osteoblasts, which could contribute to the plastron. If these results are confirmed, they indicate a shift in the differentiation potential of the late-migrating NCCs toward cell types normally derived from cranial NCCs.

To confirm the absence of neurons, I set up a positive control using chicken dorsal root ganglia (tissue composed of neurons). Dorsal root ganglia were isolated and dissociated to make a suspension of single cells. The cells in the suspension were cultured for several days, fixed, and stained with the same anti-neurofilament antibody used above. Unfortunately, these results were very poor and did not allow me to definitively rule out the presence of neurons in the preliminary experiment performed on turtle NCCs.

Despite the difficulty identifying neurons, melanocytes were readily identified because they require no staining to be seen (Fig. 1). These typical derivatives of NCCs appeared in 30% of cell groups from younger turtle embryos and in just 9% of groups from older embryos (data from 2015 and 2016). This data could indicate that NCCs from older embryos are less likely to follow the melanocyte developmental pathway.

Another major highlight was the largely successful full-scale trial. Twenty-four groups of turtle cells were stained with anti-osteopontin and anti-neurofilament (paired with a different secondary antibody in hopes of getting better results). Twenty-one of the twenty-three groups analyzed (one was damaged) contained cells that stained positively for osteopontin, indicating the presence of bone-forming cells. In addition, some cells showed tendencies to associate into structures, a characteristic of maturing osteoblasts (Declercq *et al*., 2004) (Fig. 2).

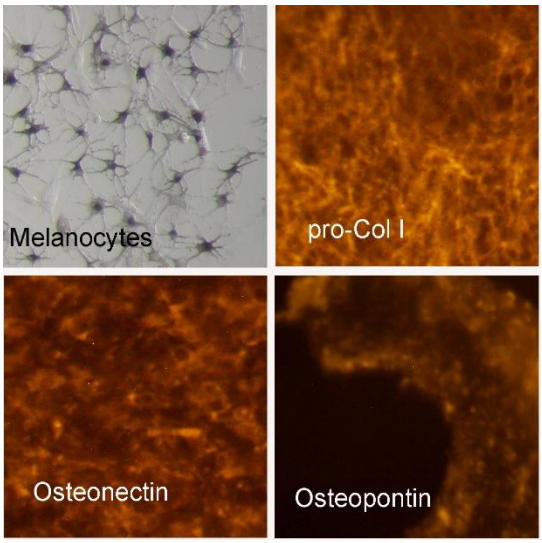

Figure 1. Photomicrographs of the melanocytes and osteoblasts cultured from late-migrating trunk NCCs. Pro-collagen I, osteonectin, and osteopontin are proteins expressed by osteoblasts, and they are visualized with fluorescent antibody staining.

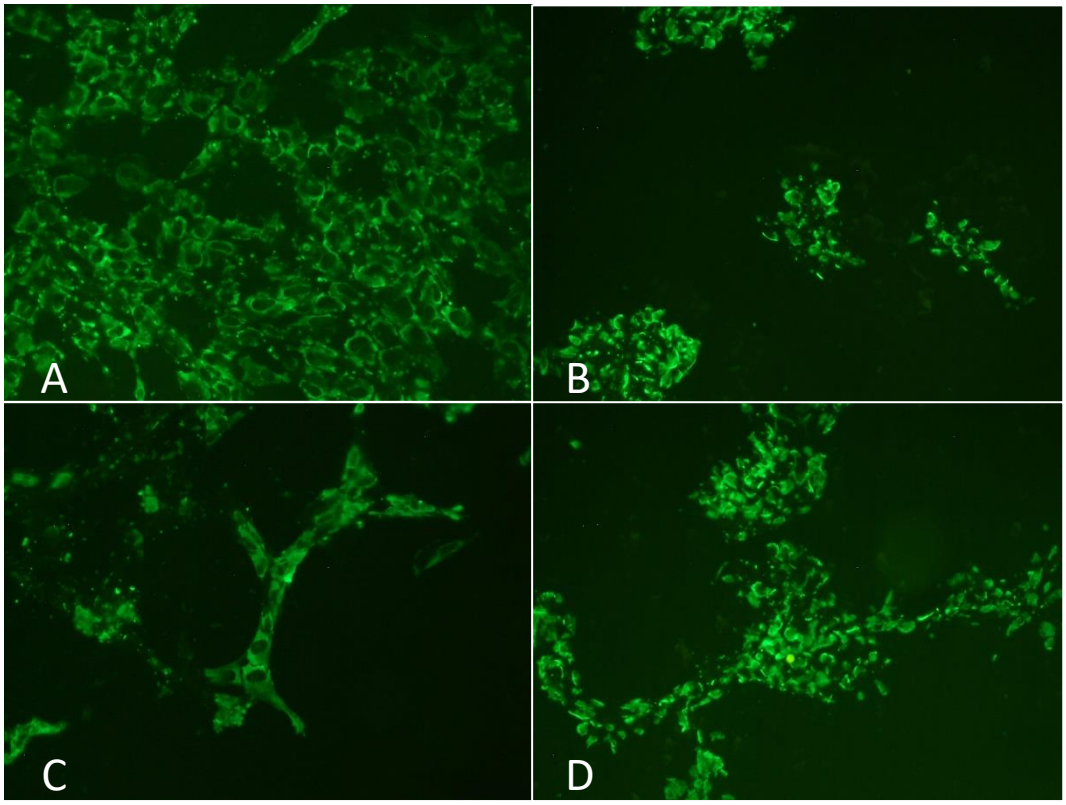

Figure 2. Photomicrographs of osteopontin-positive cells consistent with osteoblasts. A green secondary antibody was used with the anti-osteopontin primary antibody. All images magnified 100X. **A** shows a confluence of cells. Note the dark nuclei in the green cytoplasm. **B** includes several clusters of stained cells. **C** and **D** show the cells' ability to associate into structures.

Future prospects

This project will be continued during June and July of 2017 and also during the fall semester of 2017. These final two phases are 1) GFP-labeling and tracking of late-migrating NCCs and 2) examining the timing of gene expression in late-migrating NCCs. Briefly, GFP-labeling involves slipping DNA into cells and inducing them to express a gene that makes them glow neon green under UV light. Their unique coloration facilitates tracking their movements, and I will observe the migration of trunk NCCs from the spinal cord to their destination, proposed to be in the plastron. I plan to follow osteoblast differentiation by examining the expression of proteins expressed in osteoblast precursors, and during the early, middle and late phases of osteoblast differentiation.

Conclusion

In short, I was able to verify the presence of melanocytes (typical trunk NCC derivative) and cells consistent with osteoblasts (atypical derivative), but I was unable to confirm or rule out the presence of neurons (typical derivatives) due to antibody staining difficulties. Data from my project and its extensions should elucidate intramembranous ossification and thereby contribute to possible treatments for developmental maladies like craniosynostosis.

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