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Utilizing Oscillating Magnetic Fields to Manipulate Embryonic Tissues

Lynn VanderWien / Biology
Author Name/Major

Lynn VanderWien
Author Signature

8 May 2007
Date

Miles Epstein / Anatomy
Mentor Name/Department

Miles Epstein
Mentor Signature

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AUTHOR'S NAME: Lynn VanderWielen

MAJOR: Biology

DEPARTMENT: College of Agriculture and Life Science

MENTOR: Miles Epstein Ph.D

DEPARTMENT: Anatomy

MENTOR(2): NA

DEPARTMENT(2):NA

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Utilizing Oscillating Magnetic Fields to Manipulate Embryonic Tissues

Lynn VanderWielen

Abstract

Embryonic mouse tissues were incubated with gold-coated iron conjugated to a p75 antibody and then exposed to an oscillating magnetic field (OM). The p75 antibodies tag neural crest-derived cells, and via this interaction these cells can be differentially killed when treated with the OMF. These results suggest that this approach can be used to manipulate embryonic cells, but future study is required to confirm these results. Problems did occur during the experiment, and perhaps they can be explained by the conjugation process not working or possibly because the antibody/metal conjugate could not reach its destination within the tissues. Various approaches can be taken in the future to understand where problems arise and to find solutions to them.

Background

The development of the enteric nervous system (ENS) in the embryo involves the progression of enteric neural crest derived cells (ENCC) and their differentiation into neurons. Early in development, the enteric ENCC enter the pharynx, migrate, proliferate, and differentiate into neurons and glia (Conner et al 2003; Fairman, 1995). These neurons and glia are responsible for regulating motility, ion transport associated with secretion and absorption, and gastrointestinal blood flow. Most of the precursors that colonize the gut are derived from the vagal crest (somites 1-7), but the distal colon also receives precursors from the sacral crest. These crest-derived cells probably migrate as a heterogeneous population that changes with time of development. As these cells migrate, they interact with their environment to influence the outcome of their migration. Many cell signaling factors, including growth factors and elements of the extra cellular matrix, influence the differentiation of these precursors (Ratcliffe *et al.* 2004).

Distal colonic aganglionosis is caused by incomplete colonization of the hindgut by ENCC. In humans, this aganglionosis is called Hirschsprung's disease (HD), and is diagnosed soon after birth in about 1 of every 5,000 newborns. It is about four times more common in males than females (Youssef N, *et al.* 2002). HD is categorized by the absence of ganglionic cells in the distal colon. These ganglia control the coordinated relaxation of the bowel wall that is required for intestinal contents to advance. Therefore, aganglionic portions of the large intestine remain contracted and stool cannot pass. Although the region of aganglionosis varies among individuals, most often the sigmoid colon and rectum are affected.

The oscillating magnetic field (OMF) essentially heats up metal particles within the field. When tissues with gold coated iron antibody conjugates are placed into the field, only the cells with the antibodies bound to them have their cell membranes compromised and are killed. In the gut p75 is a cell surface receptor that is found only on neural crest derived cells. Therefore, when the p75 antibody is conjugated to gold coated iron and placed in the OMF, only the neural crest cells will be killed.

We hypothesized that the embryonic mouse neuronal cells would be differentially destroyed in embryonic mouse gut when tagged with gold coated iron conjugated to p75 antibodies (a neural crest cell marker). We planned to use this technique to ablate ENCC to produce a model of aganglionic gut.

Methods

Embryonic age (E) 11.5 or 14.5 mice guts from a cross between WNT-1 cre and Rosa flox Stop-Flox YFP mice were dissected under aseptic conditions in isotonic solution. This cross results in embryos in which the ENCC express yellow fluorescent protein (YFP) that can be

detected with a fluorescence microscope. The tissues were then positioned on Millipore filter paper in 2mL of Optimem media in a 35 mm plastic tissue culture dish.

For preparation of the gold-coated iron-conjugated antibodies, a fresh preparation was used for made each trial. To prepare the fresh conjugations, the isotherm of the antibody needed to be determined. A stock solution (100µg/ml) of the antibody was aliquoted in amounts varying between 5µg/ml-35µg/ml in 5µg increments together with gold-coated iron into 1.5ml eppendorf tubes. After each tube was vortexed, 10% volume NaCl was added to each tube. After another vortex, the tubes were allowed to sit undisturbed for 10 minutes, upon which a blue to pink color gradient was observed. The concentration at which the solution became pink was then the appropriate concentration of antibody to metal to be used for conjugation.

Preparation of the conjugate was done before each experiment. To conjugate the antibody, the volume of final solution for tissue incubation was determined. This volume of antibody solution was made up with the concentration found during the isotherm using 4% volume Polyethylene glycol (PEG) in the mixture and vortexed. After 10-15 minutes, the conjugated particles were centrifuged down for 30 minutes at 4,000 rpm to form a soft pellet. The supernatant was discarded and the pellet re-suspended into serum-free media. The tissues were then incubated overnight with this conjugated antibody.

After two washes with media the tissues were first photographed before incubation and then placed into the oscillating magnetic field (OMF) with a frequency of 500 kHz and amplitude of 0.7 volts. The tissues were left in the OMF in increments of 2 minutes 30 seconds, and allowed to cool for 30 seconds in-between treatments. To examine the effect of the magnetic field, the tissues were subject to the OMF for 5 minutes, 7 ½ minutes, or 10 minutes.

The tissues are then imaged again. Because the p75 antibody was conjugated to the ENCC which are YFP positive, a confirmation of antibody binding was performed. This consisted of an addition of goat anti rabbit Cy2 (1/200) secondary antibody which would bind to the p75 antibody. After overnight incubation and two washes, the tissues were then imaged again. Ethidium homodimer stain (8 μ M) was used to visualize the dead cells in the tissues.

Another route to visualize the presence of the antibodies was to utilize Scanning Electron Microscopy (SEM). After tissues were subjected to the OMF and images were taken, the tissues were fixed in 1.5% Glutaraldehyde and 1% Tannic Acid in 0.1 M Phosphate Buffer which was made fresh for fixation. The tissues were allowed to sit at room temperature for 1-2 hours, or overnight at 4°C. After fixation, the tissues were dehydrated in ethanol, for five minutes each in 30%, 50%, 70%, 80%, 90%, 95%, and 100%. The 100% ethanol soak was repeated three times. Then the tissues were inserted into the Critical Point Dryer and according to protocol, dried. To coat the tissues for preparation, they were placed in a sputter coater, and procedures continued according to standard protocol.

Images were then gathered from the SEM along with backscatter images which detect presence of metals within the tissues.

Results

Although our results could not be quantified, some trends can be seen within the data. Various trends were seen with E14.5 mouse gut tissues over the time of the 7.5 and 10 minute treatments (Figure 1). Some tissues appeared to change after the OMF treatment process, but others did not. Even within one tissue changes were not uniform (Figure 2). Some of the tissues suggest that the ENCC were differentially killed, shown by a speckled pattern of the YFP after the tissues were subjected to the OMF.

To analyze the number of cells killed in the OMF heating process, Ethidium homodimer ($8\mu\text{M}$) stain was used. A higher concentration of dead cells was seen in the control tissue that was not exposed to the OMF than tissue exposed for 2.5 minutes to OMF. However, the tissue exposed for 5 minutes to OMF showed the highest concentration of dead cells.

Discussion

Because this was a preliminary study to determine if embryonic mouse tissue could be studied using an oscillating magnetic field, numerous approaches to find an appropriate procedure for conjugation of antibody to gold-coated iron particles. The images gathered from the OMF data shows inconclusive results. Many problems could have arisen in the entire process that could explain these results.

First, a larger sample size is needed to visualize any trends in the images. Only ten E14.5 tissues were used for experimentation, and four E11.5 tissues. An oscillating magnetic field has never been used to study embryonic tissues in this way before, so for this reason it was difficult to rely on primary literature to find the most appropriate protocol or any protocol at all. This set of experiments used an antibody protocol designed for a monolayer of cell tissues, and many potential problems arose from this design. One major concern is that the antibodies are not conjugating to the colloidal gold/iron. For each animal experiment we performed an isotherm on the desired antibodies to determine the optimal antibody concentration to be used in the conjugation. This protocol never elicited a color gradient change with the antibodies, as was expected. In some of the conjugated antibodies, the pellet with the antibody did not resuspend, perhaps leading to an absence of gold-coated iron antibodies in solution available to interact with the ENCC.

To confirm the presence of the antibody coated FeAu particles, the SEM was used. This, however, needs to be perfected, because the ENCC that are targeted by the p75 antibody are within the embryonic tissues and not on the surface. In the future, perhaps tissue sections can be prepared for the SEM to look for the particle/antibody presence on the desired cells.

Another potential problem is that the antibody FeAu conjugate is not binding to the targeted cells. Because we wanted to keep the tissues alive during this process, we were not able to use detergents to allow easy access for the antibodies into the tissue. However, we have found that the p75 antibody alone can, in fact, reach the neurons without detergent (Unpublished data). This may not be the case, however, with the conjugated antibodies. The antibody conjugates are larger than the antibodies alone, and even the size of the iron-coated gold particles differ depending on how they are made. We used the same general size of particles consistently throughout the experimental process, but variations within the particles do occur. To know whether or not the antibodies are reaching the ENCC, it is possible to run a simple experiment by using a secondary antibody that has a fluorescent tag. Then, after incubation of the tissues with this secondary, if the primary antibody conjugate is in place, this will be confirmed by the presence of the secondary. We did attempt this with three tissues, but we did not obtain consistent results.

In conclusion, no concrete results can be reported due to inconsistent trends observed in the tissue, and to a low sample size. In the future, more tissues will need to be used in the experiment to draw any definitive conclusions. Possible explanations for the lack of trends were described, and from these explanations future experiments can be done to approach the specific problems, such as the use of secondary antibodies to confirm the placement of the primary antibodies.

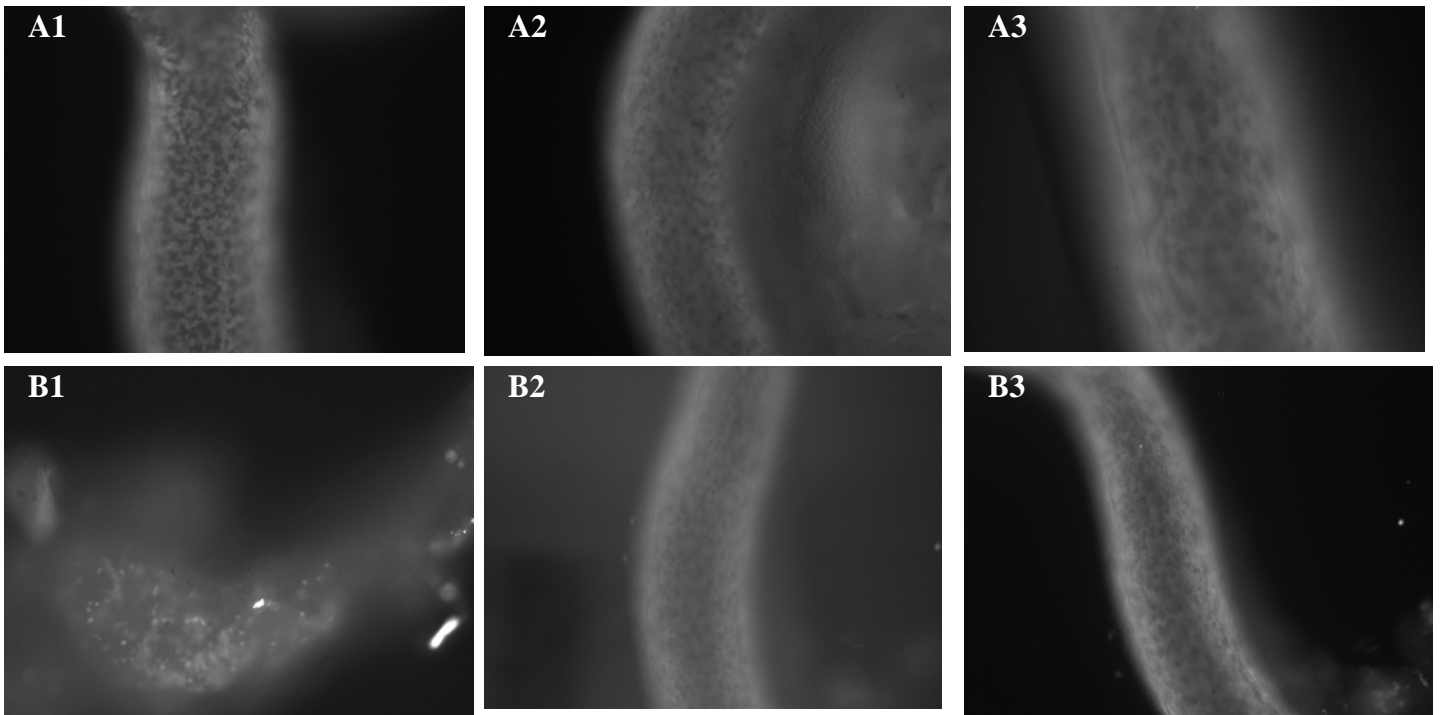


Figure 1- Two prepared E14.5 mouse gut tissues when exposed to an oscillating magnetic field (OMF) Both tissues (A and B) were prepared in the same manner and both subjected to 7.5 minutes of the OMF. Controls (A1 and B1) had a different appearance than after 5 minutes of treatment (A2 and B2) which had a different appearance that (A3 and B3), which both had the full 7.5 minute exposure to the OMF.

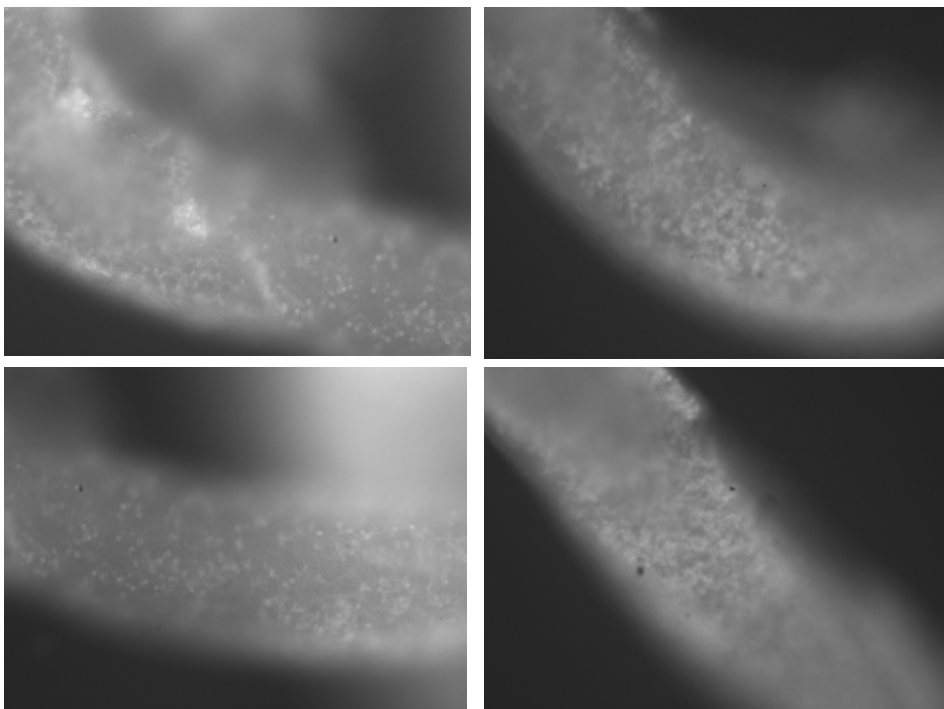


Figure 2 -Variation among single E14.5 mouse tissue after treatment with Oscillating Magnetic Field (OMF) Four different regions of the same tissue preparation that was treated with 7.5 minutes of the OMF are shown above. The number of YFP+ cells is variable between the different regions, and the YFP+ cells have a range of appearances within the tissue, suggesting the treatment had different effects on various regions of the tissue.

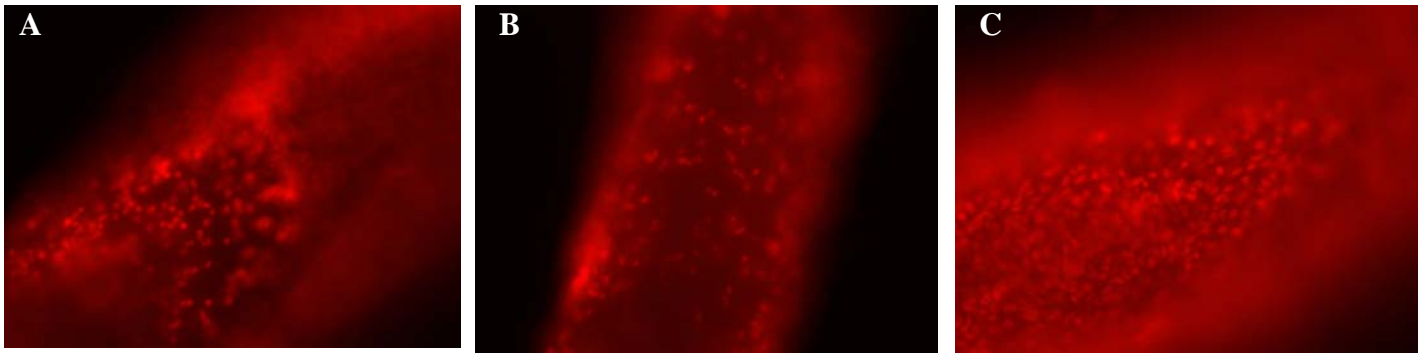


Figure 3- *Ethidium homodimer (8µM) stain of E14.5 mouse gut after Oscillating Magnetic field (OMF) treatment*
After tissues were prepared and subjected to the OMF, they were incubated with Ethidium homodimer which stains dead cells. Tissue A- not subjected to the OMF, showed more dead cells than tissue B, which was subjected to the OMF for 2.5 minutes, but fewer dead cells than tissue C, which was in the OMF for 5 minutes.

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