

## Evaluating mechanisms of nephrogenesis on tissue cultures from adult Little Skate, *Leucoraja erinacea* kidney

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The Little Skate, *Leucoraja erinacea* possesses a nephrogenic zone in the adult kidney, which resembles the embryonic metanephric kidney and contains stem cell-like mesenchymal cells<sup>1</sup>. Although understanding the underlying mechanisms of renal regeneration in *Leucoraja erinacea* is highly important, our aim was to establish a serviceable animal model for nephrogenesis. We want to utilize kidney cultures from the Little Skate and ultimately evaluate outside stimulation on this nephrogenic zone. We first evaluated differences in culturing media and established a method to evaluate these effects. We have previously shown that nephrogenic tissue survives well in culture and that proliferation of the developing stages and young tubules was vigorous as seen by the incorporation of 5-bromo-2-deoxy uridine<sup>2</sup>. We have also shown similarity between the adult skate nephrogenic zone and the developing mammalian kidney by cloning a fragment of a gene known to be important in early mammalian nephrogenesis from skate, the extracellular signaling factor Wnt4<sup>3</sup>. To evaluate the effects of different media on the nephrogenic zone of the little skate we compared the relative expression levels of Wnt4 with cultured and non-cultured kidney tissue using quantitative real-time PCR.

Small tissue pieces were micro-dissected from the kidney of Little Skate, *Leucoraja erinacea*. The first piece of tissue was flash frozen in liquid nitrogen to ensure we had a base line for each tissue piece that was an experiment. These experimental pieces were then washed in culture medium (VCM) (Valentich, J. Tissue Cult. Method 13:149-162, 1991) containing: 1:1 DME (Delbecco's Modified Eagle's Medium), 100mM NaCl, 3.9mM CaCl<sub>2</sub>, 2.5mM MgCl<sub>2</sub>, 350mM Urea, 72mM TMAO, 8mM NaHCO<sub>3</sub>, pH of 7.8 and an osmolarity of 1000 mOsm/kg. The washing medium was supplemented with 1X antibiotic antimycotic solution containing penicillin, streptomycin, and amphotericin B (Sigma). After washing, the tissue pieces were transferred into sterile six well tissue culture plates with 5 ml of medium. The first medium (V) contained VCM only. The second medium (VI), 10X Insulin-Transferrin-Sodium Selenite liquid media supplement (ITS, Sigma) was added to VCM. Medium three (VS), filtered shark serum was added to VCM. The fourth medium (VIS), both ITS and shark serum was added to VCM. The plates were placed at 17°C in a Thermo Forma CO<sub>2</sub>/O<sub>2</sub> incubator with a water jacket and a re-circulating refrigerated water bath and the tissues were gassed with 1% CO<sub>2</sub> and ambient O<sub>2</sub> levels. The medium was changed every 48 hours. After one week the cultures were flash frozen in liquid N<sub>2</sub>. RNA was extracted using Trizol, Dnase Digested (Ambion) and transcribe into cDNA (Promega). We utilized the ABI Prism 7700 Taqman Real-Time PCR to analyze the relative mRNA expression. Specific primers from Wnt4 were previously designed and tested. The amount of cDNA in the PCR reaction was quantified using SYBR Green chemistry (Invitrogen). For normalization of the results, the expression of B-actin was used as a reference point. Standard thermal cycle conditions for the ABI Prism 7700 Taqman were followed. The fluorescence intensity was generated in real-time data during PCR amplification. The data was then imported into an Excel spreadsheet and analyzed.

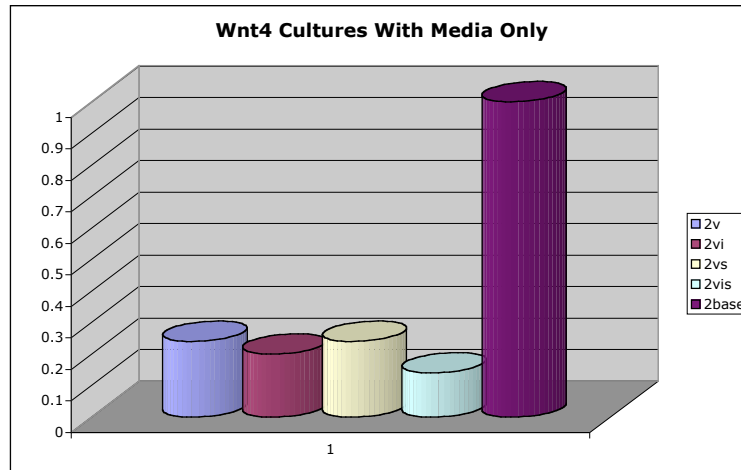


Fig 1. Real-time PCR data of Wnt4 expression from skate developing kidney cultures in different culture media. 2V: VCM media only, 2VI: VCM plus ITS, 2VS: VCM with shark serum, 2VIS: VCM media with ITS and shark serum 2Base: Base line was frozen tissue not cultured.

Our preliminary data showed that there is very little difference between these four media. The data also showed the highest level of expression of Wnt4 in the baseline tissues. Since the expression of Wnt4 decreases after it is placed in media that has been shown to sustain kidney tissue other genes should also be investigated. In conclusion, we are able to assess effects on tissue cultures using real-time PCR and specific gene expression levels.

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