Summer Student Vacation studentship SRF Report

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During my 6 week vacation studentship in Tom P. Fleming's lab at Southampton General Hospital, University of Southampton, I was supervised in the laboratory by PhD student Congshan Sun as well as Tom P. Fleming, to develop my lab skills and aid in my research. Congshan's work had provided evidence that by changing the in vivo environment of the preimplantation embryo via maternal low protein diet it has been observed that the endocytosis system was stimulated in the blastocyst trophectoderm. The specific aims of my work performed during my placement were;

1. To learn how to collect and culture mouse embryos
2. To experience the technique of 'real time' PCR to quantify embryo gene expression
3. To learn the techniques of immunocytochemistry and confocal microscopy to assess localisation of endocytosis receptor megalin in blastocyst trophectoderm cells
4. To learn the technique of immunoblotting to detect and quantify endocytosis related proteins in tissue samples
5. To conduct an experimental analysis of environmental protein level to see whether different Bovine Serum Albumin (BSA) concentrations within in vitro culture media modified the endocytosis system of preimplantation mouse embryos, by testing FITC-dextran endocytosis levels.

1. I learnt the correct dissection technique of mice, specifically the removal of the oviducts to perform flushing of 2-cell or later stage embryos from E1.5 or E.3.5 which were to be used in later experiments and training. I learned to flush MF1 mouse oviducts under a dissection microscope and collect embryos via finely drawn pipettes (made from glass Pasteur pipettes). Collected 2-cell embryos were cultured at 37^{o}C in KSOM media and my embryos developed normally.

2. Real Time PCR is used to quantify gene expression level. It begins with RNA extraction via the use of Dyna-beads. Embryos are treated with lysis buffer and the resulting lysate is placed into solution with Dyna-beads which bind the mRNA. This mRNA is then eluted from the beads after washing and is used to produce cDNA via a reverse transcription reaction. This cDNA is then used in real time PCR to detect gene expression. Genes of interest are tested for by using specific selective primers, I was looking for megalin gene expression using Meg1 primer.

3. Immunocytochemitry; embryos were first treated with acid Tyrodes to remove the zona pellucida, then washed in culture medium before fixation in paraformaldehyde, washing in PBS, permeabilising with Triton-X100, application of primary antibody (eg, megalin), another wash, application of secondary antibody with fluorescence tag, wash again, including nuclear dye (propidium iodide) and mounting in anti-fadant medium. Controls included

omission of primary antibody. Embryos were analysed using a SP5 confocal microscope for distribution of megalin and other antigens. Images were analysed by Volocity or related software.

4. Immunoblotting (or Western Blotting) is used to detect protein from tissue lysate, in our case yolk sack lysate. The first step was gel electrophoresis, to separate the proteins in the samples; different lysates and a heart lysate control were run on a pre-cast gel alongside a commercial MW ladder to compare bands against. A transfer was then set up where the proteins from the gel are transferred to a membrane. Transfer cassettes were produced and placed into a transfer tank so that the gel face the positive pore and membrane faces the negative pore. Once the transfer is completed, the membrane is treated with primary antibody which binds the protein product of interest during overnight incubation at 4^{o}C. Secondary antibody is then attached using secondary antibody + milk buffer solution to bind to the primary antibody. The membrane is then imaged using a Licor Odyssey Infra-red detector.

5. The main experiment was an analysis of the effect of environmental protein level on trophectoderm endocytosis. Collected 2-cell embryos were cultured at 37^{o}C in 3 different KSOM media containing varying concentrations of BSA, either 1 mg/ml or 4 mg/ml, until they reached the blastocyst stage on day 4. Once embryos were at the blastocyst stage, they were incubated in 0.5 mg/ml FITC-Dextran medium (which also contained the level of BSA they were incubated in) for 1 hour. Another experiment was run alongside whereby a 'switch' incubation of culture medium was conducted. Here, 2-cell embryos were once again cultured in either 1 mg/ml or 4 mg/ml BSA KSOM media, but now after the initial incubation up to the blastocyst stage they were incubated for 1 hour in the other KSOM media (i.e. in 4 mg/ml BSA would be replaced by 1 mg/ml and vice versa). This incubation was for just 1 hour then they were incubated in 0.5 mg/ml FITC-Dextran medium which contained the level of BSA they had been changed into for the last hour.

After incubation was finished, the zona pellucida was removed using acid Tyrodes and blastocysts were fixed. The fixed embryos were then imaged using a confocal microscope. Leica SP5 confocal was used and the z-series was 1 µm per photograph (optical section). Figure 1 shows an example confocal image. This blastcocyst is from the switch medium experiment, it was swtiched from 1 mg/ml BSA to 4 mg/ml in the last hour and shows a high level of FITC- Dextran staining.

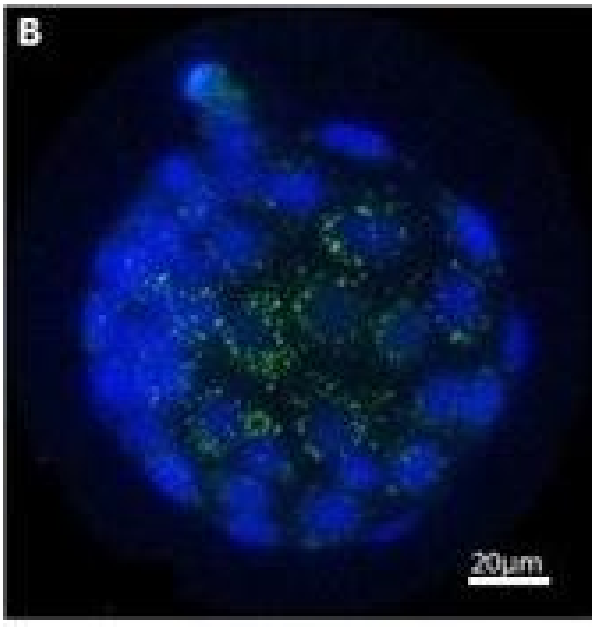

Figure 1 – Confocal image showing FITC dextran staining

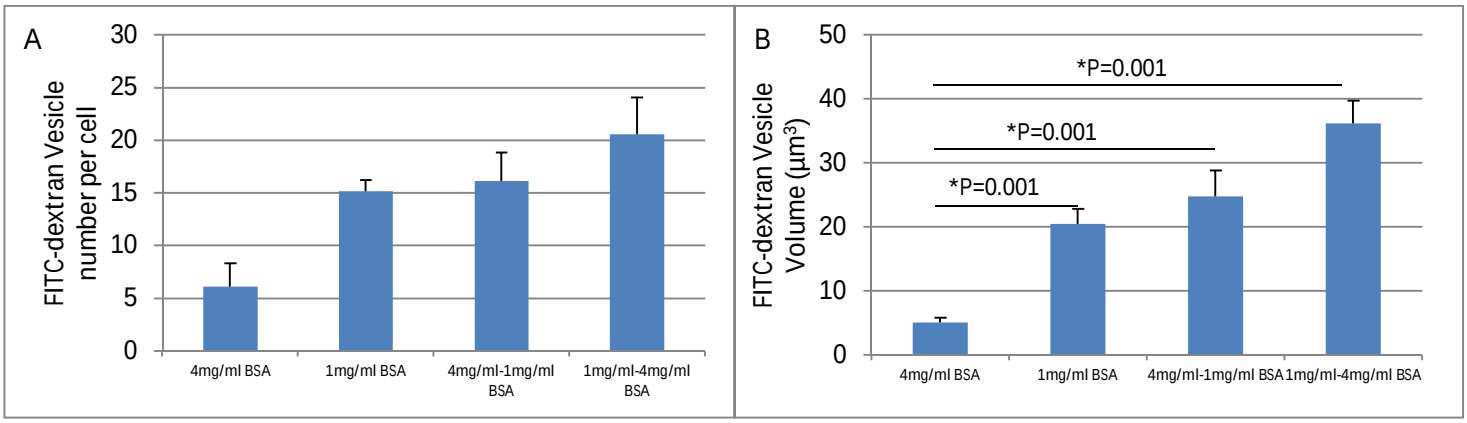

Figure 2 – Results of FITC-dextran vesicle total volume and number per cell in Blastocysts across the single medium and switch medium experiments.

Figure 2 shows the results of the confocal microscopy after analysis with Volocity software completed by Congshan Sun after all repeats were finished. The total number and volume of FITC-dextran vesicles from images like figure 1 were counted and then divided by the number of nuclei to determine the values per cell. Figure 2A shows that there was no statistical difference between the numbers of vesicles endocytosed although a clear trend is visible making it appear that the level of endocytosis may increase in lower levels of BSA, particularly when involved in switch medium experiments.

However a statistical difference was shown for the volume of FITC-dextran vesicles per cell (fig 2B) between 4 mg/ml BSA and the 1 mg/ml BSA treatment. This indicates that local protein level in the embryo environment is sufficient to stimulate endocytosis activity to compensate. In the 4 mg/ml to 1 mg/ml switch experiment, endocytosis increased to equivalent levels from the 1 ml group, indicating a short duration of reduced protein exposure is sufficient to stimulate increased endocytosis. In the 1 mg/ml to 4 mg/ml switch experiment the endocytosis activity was equivalent to or higher than (although not significantly different) the 1 mg/ml group. This indicates that once high endocytosis is stimulated by 1 mg/ml protein conditions, the stimulation is not reversed by returning the blastocyst to high protein levels.

Collectively these patterns of endocytosis detected through environmental change in vitro support the concept from our in vivo work that poor maternal protein level in diet acts to stimulate endocytosis and once stimulated, the level of endocytosis remains high regardless of a further change in environmental protein level.