Biochemical Society Summer Vacation Studentship Report, Summer 2009 (Eisenthal Prize Winner)

Student: Christien Buchwald (Supervisor: Dr. Kathryn Taylor)

Project title: ***Examination of the functional role of ZIP7 phosphorylation in the ZIP7 dependant zinc wave***

Description of project / Background

Zinc is essential for a diverse range of cellular functions such as gene transcription, growth, differentiation and metabolism. Both deficiencies and excesses of cellular zinc are detrimental to health. An excess of intracellular zinc can lead to exaggerated cell growth and migration of cells, and therefore is implicated in breast cancer. There have been shown to be stores of liable zinc present in intracellular membrane structures and a small and fluctuating but biologically significant pool of liable cytosolic zinc. Zinc signalling has both genomic and much faster non-genomic components; it can inhibit protein tyrosine phosphatase activity, resulting in activation of MAP Kinases (such as ERK1/2, JNK, p38) and tyrosine kinases Src and EGFR which rely on dephosphorylation for their inactivation.

ZIP7 is part of the ZnT family which transports zinc out of its intracellular storage compartments such as the endoplasmic reticulum. This results in activation of multiple tyrosine kinases through zinc-mediated inactivation of protein phosphatases. Breast cancer cells have elevated levels of ZIP7. It is completely unknown how ZIP7 functions but a whole genome phosphorylation screen showed that ZIP7 was phosphorylated on S275/S276. Both these residues are predicted to be phosphorylated by a Kinase which I shall refer to as Kinase X.

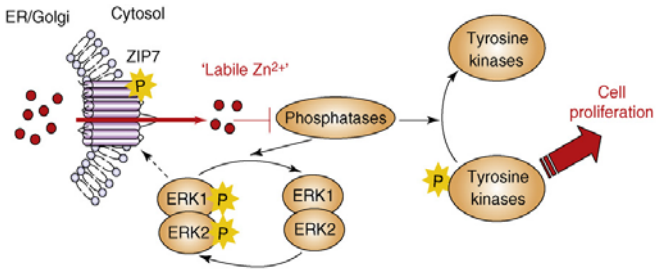


Figure 1. Zinc Wave. *Left is a diagram which shows cytosolic zinc wave causes inhibition of phosphatases, resulting in a shift of tyrosine kinases to their phosphorylated and active states which itself can lead to uncontrolled cell growth and anti-hormone resistance.*

(Hogstrand C, Kille P, Nicholson RI, Taylor KM (2009) Zinc transporters and cancer: a potential role for ZIP7 as a hub for tyrosine kinase activation, Trends Mol Med. ***15****(3), 101-111.)*

Aims

1) To confirm the association of ZIP7 and Kinase X and to provide temporal information (by gel electrophoresis, immunoprecipitation and western blot) which could be related to the generation of the zinc wave, suggesting whether phosphorylation is inhibitory or activating.

2) By fluorescent microscopy, reproduce the temporal zinc treatment to observe any change in cellular location of different molecules during the generation of the zinc wave.

3) Confirm the presence of downstream molecules by western blot and the effect of a Kinase X inhibitor.

Departures from original proposal

There were no major departures from the initial plan. Although FACS was carried out, results are not included as the experiment was inconclusive and there was no time to repeat it. However, because Kinase X was implicated in the zinc wave mechanism there was an opportunity to also investigate the effect of a Kinase X inhibitor on the activation of downstream molecules of the zinc-wave, such as AKT and ERK providing additional functional information.

Immunoprecipitation with ZIP7

I carried out 4 total experiments immunoprecipitating (IP) TamR cells with ZIP7 and probed resulting Western blots for Kinase X to detect any association of ZIP7 and the Kinase X over a time course of zinc treatment. This was sufficient to generate the ZIP7-dependant zinc wave.

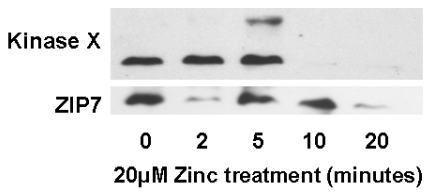


Figure 2. Immunoprecipitation with ZIP7*. A representative result from one IP experiment which shows an increase in intracellular zinc at 2 minutes following normalisation after probing with ZIP7 and carrying out densitometry.*

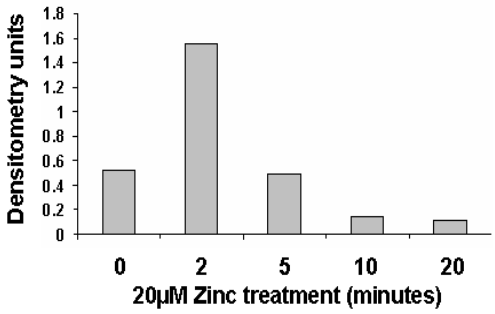


Figure 3. Average densitometry normalisation data*. A graph pooling the average densitometry normalisation data from all 4 IP experiments shows an average increase in intracellular zinc at 2 minutes.*

To confirm these findings, the experiment was reversed and the Kinase X antibody was used for immunoprecipitation, probing the Western blot with ZIP7 antibody. These results show that Kinase X and ZIP7 were associating, peaking on average after 2 minutes of zinc treatment. I also imunoprecipitated with Kinase Y (another related kinase) and probed for ZIP7 on the Western blot, this showed a stable level of Kinase Y associated with ZIP7 from 0-20 minutes and therefore, I conclude that it is not involved with ZIP7 during the zinc wave. To confirm the action of Kinase X on ZIP7 I probed two blots (not IP), both for activated ERK and AKT, with and without pre-treatment with the Kinase X inhibitor. The results showed that when treated with Kinase X inhibitor the activation of both these downstream molecules decreased and therefore, further confirmation that Kinase X is involved in the zinc wave.

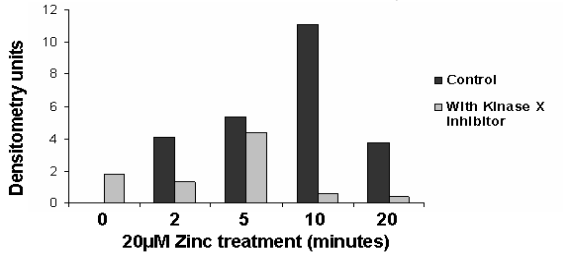
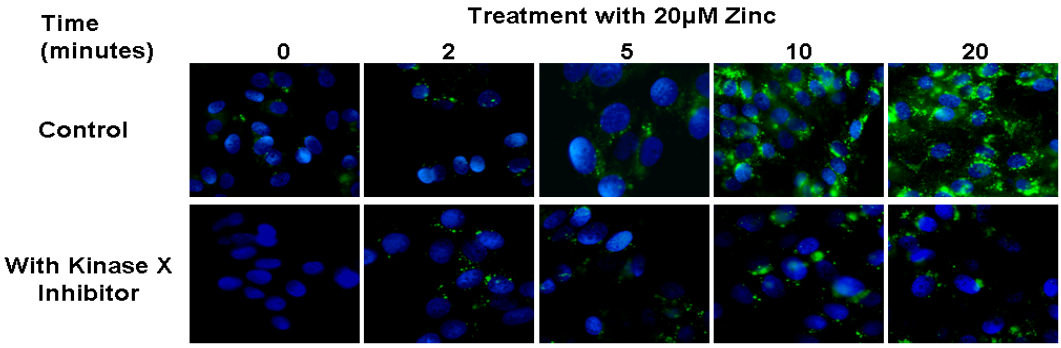


Figure 4. Densitometry normalisation data. *A graph containing densitometry normalisation data after probing Western blots for pERK.*

Fluorescent Microscopy

Figure 5. TamR cell Zinc Wave. *Pictures of TamR cells that have been treated with 20μM zinc + pyrithione for 0-20 minutes, nucleus stained blue with DAPI and intracellular zinc stained green with FluoZin-3©.*



Two fluorescent microscopy experiments were conducted where TamR cells were treated with zinc over a time period of 0-20 minutes. The cells were stained with various dyes, including Zinquin and FluoZin-3© which both bind to and detect zinc. During these levels of zinc were negligible at 0 minutes, slightly increasing at 2 and 5 minutes but increasing drastically at 10 minutes and remaining high at 20 minutes. However, in the presence of the Kinase X inhibitor, levels of zinc at time 0 were again negligible, increasing slightly at 2 minutes but much reduced compared to control at 5-20 minutes, suggesting that Kinase X may phosphorylate ZIP7 of the Serine residues (mentioned above) and is therefore responsible for the increased levels of intracellular zinc by release from the endoplasmic reticulum during the zinc wave in TamR breast cancer cells. This is an exciting result that now needs further investigation.

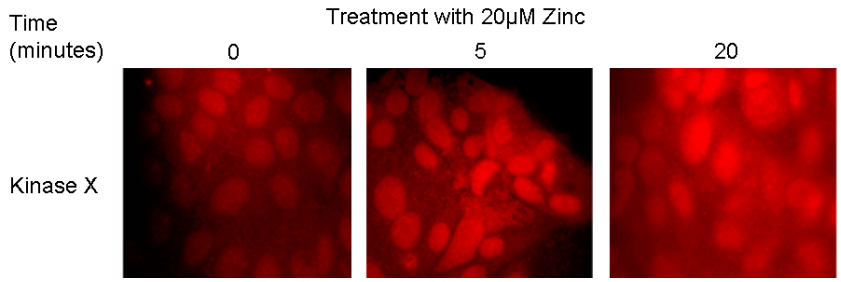


Figure 6. Cellular Distribution of Kinase X. *TamR cells treated with 20μM zinc + pyrithione for 0-20 minutes, Kinase X stained red with Alexaflour-594. Initially Kinase X appears more cytoplasmic, by 5 minutes it appears nuclear and by 20 minutes it seems to have dispersed throughout the cell.*

Value of the studentship to the student

My first experience of working in a research environment was very enjoyable, rewarding and hugely beneficial. I found the placement extremely stimulating and educational, strengthening my existing biochemical knowledge and providing experience of a range of laboratory techniques (cancer cell culture, cell harvesting, centrifugation, densitometry, protein assays, gel electrophoresis, Western blotting, Fluorescent microscopy, FACS, image processing). I also learned the importance of key skills such as time management, health and safety, planning experiments ahead of carrying them out and keeping a detailed lab book of all procedures completed. The experience has been good practice and a confidence boost for my lab-based research dissertation which I will be undertaking this year. My desire to work in biomedical research has been further stimulated and I have begun to search for PhD projects.

Value of the studentship to the lab

This studentship has been extremely beneficial to my lab, providing the opportunity to confirm a previous hypothesis and provide sufficient preliminary data for a grant proposal. This hypothesis suggested that a kinase was involved with a zinc transporter, ZIP7, and specifically in the generation of the intracellular zinc wave which has been demonstrated to be a likely cause of progression of some breast cancers. The involvement of a kinase is an exciting and previously unprecedented discovery that now warrants further examination as a means to target aberrant breast cancer growth.