Optimising a C2C12 alkaline phosphatase assay

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Aims

To develop an assay that provides an indication of the BMP signalling within cells following treatment with inhibitors of epigenetic targets. Alkaline phosphatase (ALP) is a protein that is upregulated following stimulation of the BMP signalling pathway and is thus commonly used as a readout for BMP signalling activity. This assay uses the ALP substrate, 4-methylumbelliferyl (4-MUP), to generate a fluorescent signal proportional to the ALP activity in the C2C12 population.

Procedure

20180529
110 C2C12 cells were seeded per well in a 96 well plate in enough wells for 3 technical repeats of a BMP-7 ligand titration.

(This cell number was previously optimised for 7 days of growth in culture without reaching over-confluence, however I suspect that the proliferation of the C2C12 cells increased with each passage. Thus, because these cells were one passage older, they were over-confluent by the end of the experiment).

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Three days of ligand treatment: Growth Medium (DMEM, 10% FCS, gentamycin) was removed from cells and replaced with GM containing 600, 400, 200, 100, or 0 ng/mL BMP-7 (three technical repeats each).

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One day of ligand treatment: As above, medium was removed and replaced in a final technical triplicate and replaced with GM containing 400 ng/mL BMP-7.

(This time point was included to test whether the time in the presence of the epigenetic probe can be made as long as possible before testing the change in BMP signalling activity, within the 7 day growth window available).

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Cells were harvested, and ALP activity measured as follows:

1. Remove 10uL medium from each well
2. Add 10uL PrestoBlue to each well (manufacturer's recommended volume)
   (PrestoBlue signal allows normalisation of results the number of viable cells present in each well).
3. Incubate 2h, 37oC
4. Read fluorescence (ClarioSTAR excitation 535-20nm, emission 580-20nm)
5. Wash 3x with PBS (leave empty after last wash).
6. Add 25uL CellLytic M (with 1 Roche mini-protease tablet per 10mL) to each well
7. Incubate for 15min, 22oC, then shake 10Hz (600rpm) for 30s
8. Add 100uL 3:1 4-MUP:Tris (50mM pH 8)
   (NB this maintains a 3:1:1 ratio of CellLytic M:4-MUP:Tris)
9. Read plate (Ex 340-20nm, Em 520-20nm, ClarioSTAR) immediately (T0), after 30min, and after 60min (with incubation at 37oC between each read)

Results

A maximum fold change of ALP activity of 2.97 was observed following 3 days of treatment with 600ng/mL of BMP-7 (below). One day of BMP-7 treatment generated a 1.2 fold increase in ALP activity (below), however as this is so much lower than the 3 day time-point, this treatment will not be selected. In future cells will be treated with 400ng/mL of BMP-7 as there wasn't much increase in signal above this concentration. As the fold change relative to the untreated control increased between the 30 and 60 minute incubation time-points, later time-points will be included in future to test whether the fold change increases further.