Dr Alicia D'Souza
Institute of Cardiovascular Sciences
3rd Floor Core Technology Facility
46 Grafton Street
Manchester M13 9NT
Tel:+ 44(0)16127551207
Email:Alicia.dsouza@manchester.ac.uk

ESC First Contact Initiative Grant- Report

Recipient: Dr. Alicia D'Souza, Research Fellow, University of Manchester, Manchester, UK
Host: Dr. Paula Da Costa Martins, Assistant Professor, CARIM School of Molecular Cardiology, Maastricht University, Netherlands
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BACKGROUND

Athletes are prone to cardiac arrhythmias. They have sinus bradycardia and a higher than normal incidence of sick sinus syndrome, electronic pacemaker implantation, heart block and bundle branch block[1-3]. Recently, we have shown that sinus bradycardia, the most common 'arrhythmia' in athletes, is attributable to electrophysiological remodelling of the sinus node, the pacemaker of the heart, and not high vagal tone as commonly believed[4]. We demonstrated a training-induced downregulation of ion channels in the sinus node including the key pacemaker channel HCN4 that carries the pacemaker current I_f. An important future direction for this work is to identify transcriptional regulatory circuits that underlie the dysregulation of ion channels in the trained heart, with a view to reversing training-induced arrythmogenic remodelling. In previous work we reported that there was a downregulation of the transcription factor *Tbx3* and an upregulation of *NRSF* in the trained sinus node[4]. We now wish to further investigate whether *Hcn4* is a direct transcriptional target of the aforementioned transcription factors and whether their interaction is altered by endurance exercise. To this end, the goal of this visit was to obtain skills in the techniques of **electrophoretic mobility shift assay** (EMSA) and **chromatin immunoprecipitation** (ChiP) in order to set-up these techniques in my laboratory in Manchester. Both techniques are central to studying gene regulation and determining protein:DNA interactions.

EXPERIMENTAL DETAILS

***In Silico* Analysis:** I was initially familiarised with concepts in transcription factor regulation of genes and theoretical considerations in performing both the EMSA and ChiP assays. During this time I also learned methods in *in silico* analysis of promoter regions including using Ensemble to retrieve the 5' flanking region and coding sequence of the HCN4 gene. Putative transcription factor binding sites were predicted using the rVista function within the ECR browser (http://ecrbrowser.dcode.org/). Using this technique I was able to identify four evolutionarily conserved binding sites for NRSF binding neuron-restrictive silencing element (NRSE) in the HCN4 gene (**Fig.1**).

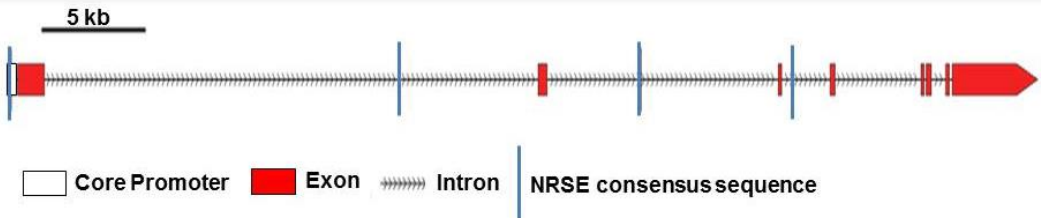

Figure 1. Conserved NRSE binding sites in mouse *HCN4*

2. EMSA: I then performed an EMSA using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific). For the purposes of training, we utilized the EMSA optimization and control kit comprising reagents for setting up and customizing DNA binding reactions and a control set of DNA and protein extract to test the system. Briefly, we used manufacturer provided biotinylated- Epstein barr nuclear antigen (EBNA) control DNA and EBNA extract to demonstrate a functional EMSA assay (**Figure 1**). Unlabelled EBNA DNA served as the positive control. Reactions were prepared as per the manufacturers instructions, electrophoresed on a 6% PAGE gel, transferred to a nylon membrane, and crosslinked using UV light. Biotin-labelled DNA was detected using a cooled CCD camera that captures light emitted (when the luminol-based substrate is converted to an excited intermediate dianion by horseradish peroxidase in the presence of hydrogen peroxide). Altogether it was the ideal introduction to the technique and showed me the stages involved in developing a working assay and the processes used to confirm binding interaction specificity.

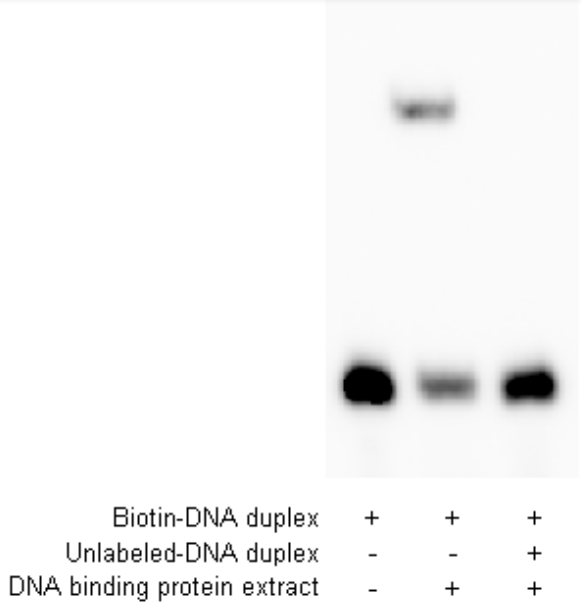

Figure 2. Functional EMSA. Specific DNA:protein interaction demonstrated by shift of Biotin labeled DNA+EBNA protein extract (lane 2). Signal shift is prevented by competition from excess unlabeled DNA (lane 3).

3. ChiP: The rest of the visit was focused on setting up and performing an *in vitro* ChiP assay. Bioinformatics analysis (i.e. those described in section 1) demonstrated a conserved Hand2-specific E-box binding site in an 8kb upstream promoter region of *Calm3*. (**Fig 3**) The aim of the experiment was to validate the regulation of *Calm3*

by *Hand2 in vitro* using ChiP. I systematically performed the DNA-protein cross-linking, cell-lysis, chromatin shearing, immunoprecipitation, crosslinking reversal, DNA isolation and qPCR quantitation steps involved as follows:

3T3L1 cells were seeded at 5000 cells / cm^2, infected with AdHand2 and compared to un-infected cells. Cells were crosslinked with 1% formaldehyde at room temperature and stopped after 10 min using 125 mM glycine. After washing 3 times with ice-cold PBS cells were lysed in a buffer containing 5 mM PIPES at pH 8.0, 85 mM KCl and 0.5% NP-40 containing protease inhibitors. Nuclei were pelleted by centrifugation and lysed in 50mM Tris at pH 8.1, 10mM EDTA, 1% SDS and protease inhibitors as above. Extracted DNA was sonicated on ice to fragments of 0.3–1.5 kb using 20 s bursts (up to a total of 2 minutes/sample) on a Branson Sonifier 250. After clearing debris by centrifugation, samples were diluted fivefold in 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris and 167 mM NaCl containing protease inhibitors and pre-cleared with 80 µl salmon sperm/protein A agarose slurry for 30 min at 4 °C with rotation; 20% of the pre-cleared lysate was saved as gDNA input control. Beads were pelleted and supernatant incubated with either 5 µg of rabbit polyclonal anti-Hand2 (Santa Cruz, sc-22818) or isotype control IgG. Immune complexes were collected with 60µl of salmon sperm DNA/protein A agarose slurry for 1 h at 4 °C with rotation. Beads were washed sequentially in TE buffer containing **i**. low salt (150 mM NaCl), **ii**. high salt (500mM NaCl) and **iii**. LiCl. Complexes were then eluted by vortex in 250µl of buffer contaning 1% SDS 0.1 M NaHCO3 for 15 min. Formaldehyde crosslinks were reversed by overnight incubation with RNase and NaCl at 65 °C for 5 h. DNA was then concentrated by ethanol precipitation and resuspended in water with 2 µl of 0.5 M EDTA, 4 µl of 1 M Tris at pH 6.5 and 1 µl of 20 mg ml^{-1} proteinase K for antibody digestion at 45 °C for 2 h. Finally, DNA was purified by application to QiaQuick spin columns (Qiagen) and PCR was carried out using specific primers for *Calm3 (*Forward: GGAGATGGCACCATTACCAC, Reverse: TCGTATCTCCTCCTCGCTGT).

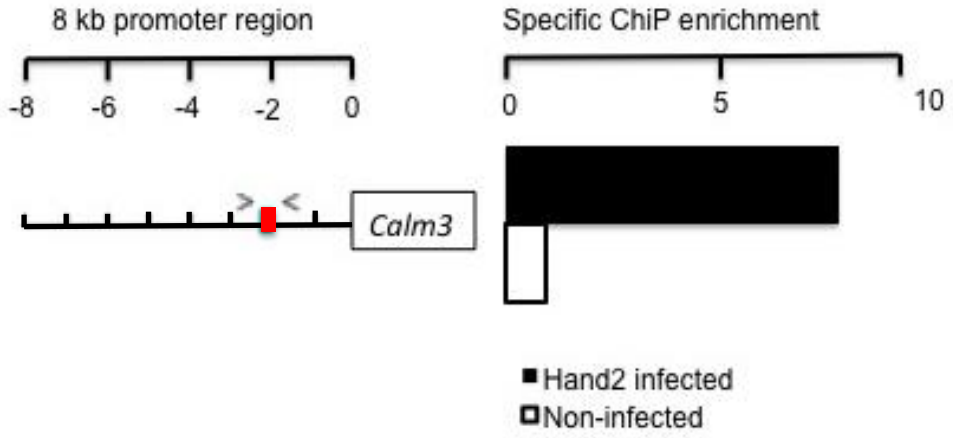

Figure 2: Upstream region of Hand2 target gene Calm3 and position of primers flanking Hand2-specific E-box (red box) used in Hand2 ChIP assay. Relative ChIP enrichment with *Hand2* (normalised to ChIP gDNA input control) is indicated in 3T3L1 cells infected with AdHand2; Gene expression is normalised to un-infected condition, set at 1-fold.

FUTURE DIRECTIONS

In the very near future, I hope to set-up the techniques outlined in this report in my lab at Manchester with guidance from the Da Costa Martins lab. This will be a critical step in investigating the mechanisms controlling the electrophysiology of the cardiac conduction system in health and disease. This visit also marked the start of a collaborative research program between Manchester and Maastricht. We identified converging research interests on other projects and have begun collaborative experiments on the same.

ACKNOWLEDGEMENTS

I am grateful to the European Society of Cardiology for this grant which facilitated a wonderfully stimulating lab visit. My very great appreciation is extended to Dr Paula Da Costa Martins for her continued guidance and support. Special thanks are due to Mr. Servé Olieslagers for his time and patience in the lab.

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