

HypoxiaNet (COST Action TD-0901-141110-003831)

STSM report-Analía Nuñez O'Mara

From 14th to 27th November 2010, I joined Dr Peter Staller's laboratory for a training period. The aim of this short-term mission was to learn the basis of the Chromatin Immunoprecipitation assay (ChIP), technique that is useful to study DNA-protein interactions. I was particularly interested on the study of the HIF-DNA binding capacity. HIF (Hypoxia Inducible Factor) is activated by reduced oxygen availability and this activation is essential to maintain oxygen homeostasis and to assure cell survival. By the selective binding to HRE (Hypoxia Response Elements) sequences, HIF regulates the expression of a number of genes that are responsible for cell survival under hypoxic conditions. Inadequate HIF-target genes expression results in deficient adaptation to hypoxia and contributes to disease pathogenesis. Therefore, the analysis of how the HIF-DNA interaction is modulated could be helpful in the study of several diseases where HIF is involved, such as ischemia and cancer.

This STSM offered me an excellent opportunity to learn from Dr Peter Staller's expertise. The ChIP protocol has several steps and it requires between 4 to 6 days to complete the whole procedure. Herein, I have summarized all the different steps that were performed during the training period.

❖ *ChIP in HeLa cells*

We first performed the ChIP assay in HeLa cells. These cells have been used in Dr Staller's laboratory as a model system, therefore the working conditions were optimized for this cellular model.

Cell Culture:

HeLa cells were plated in 15cm dish, one plate for each ChIP, to be at 80% confluent when were split. Cells were incubated in normoxia or hypoxia. For the hypoxic treatment, cells were exposed to 0, 5% O₂ in an hypoxic incubator (Ruskin, "In vivo" 400) during 6 hours.

Crosslinking:

The DNA-protein complexes need to be covalently linked to perform the ChIP. The crosslinking step was performed by adding formaldehyde (final concentration: 1%)

directly into the cell culture medium. After 10 minutes shaking at room temperature, the crosslinking was stopped with the addition of a Glycine solution (final concentration: 125mM) for 5 minutes.

Collection, lysis and DNA fragmentation:

Then, the cells were washed with cold PBS [Phosphate Buffered Saline: NaCl 155mM, Na₂HPO₄ 7H₂O 2.97mM, KH₂PO₄ 1.06mM], and scrapped down also with the PBS solution. Cells were collected by centrifugation, and lysed with SDS buffer [1% SDS, 100mM NaCl, 50mM Tris-Cl pH 8.1, 5mM EDTA pH 8.0, 0.02% NaN₃]. After a short centrifugation, the pellet was resuspended in the IP buffer [(2:1) SDS lysis buffer + Triton dilution buffer (5% Triton-X-100, 100mM Tris-Cl pH 8.6, 100mM NaCl, 5mM EDTA pH 8.0, 0.02% NaN₃), and protein inhibitor cocktail].

At this point, we have the proteins that bind to DNA (under the different experimental conditions we used) covalently linked to the chromatin. To reduce the unspecific signal and to improve the ChIP assay resolution, the DNA was fragmented in small pieces (below 500pb) before performing the immunoprecipitation. For that, the extracts were sonicated in Diagenode water bath sonicator using a defined energy level and time, and then the quality of the fragmentation was analyzed in an agarose gel, as it is shown in figure 1. The cell extracts were frozen at -20°C.

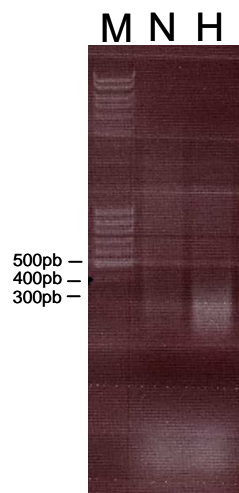


Figure 1: Agarose gel of HeLa cell extracts after sonication. M: DNA Marker. N: Normoxia. H: Hypoxia.

Pre-clearing:

To reduce the unspecific signal, the extracts need to be pre-cleared with the sepharose A or G protein beads that are used after the antibody-extracts incubation to capture the antibodies. The using of the A or G protein beads depends on the antibody that it is using; e.g.: monoclonal antibodies link to G protein, and polyclonal antibodies to A protein. In this case, we pre-cleared the cell extracts with 40 μ l sepharose A protein beads (1:1 diluted in IP buffer) that were previously blocked with herring sperm and BSA.

Protein quantification:

After the pre-clearing, the cell extracts were quantified by the Bradford method and the proteins were diluted to 1mg/ml in IP buffer. At this point, an input aliquot is reserved to later use as reference value.

Cell extracts-Antibody incubation:

At this step, the proteins of our interest, that are covalently linked to specific DNA fragments, will be recognized by specific antibodies.

1mg of cell extracts was incubated with the following antibodies:

- IgG (2 μ g antibody, rabbit serum, SIGMA I8140): Used as a negative control.
- Anti-H3 (Histone H3; 15 μ g antibody, Abcam ab1791-100): Used as a positive control
- Anti- HIF1 α (20 μ l, Abcam ab 2185)

The cell extracts-antibody mixture was incubated on rotation wheel overnight at 4°C.

Beads-Antibody-Protein-DNA capture:

The Antibody-Protein-DNA complex was pull down to selectively capture only the complex of our interest and remove all the protein-DNA elements that we are not interested in. To do that, the cell extracts were first centrifuged at high speed to remove aggregates that had accumulated over night, and then incubated with 40 μ l of pre-blocked sepharose A protein beads (see above) in rotation wheel for 3hs at 4°C.

After the Beads-Antibody-Protein-DNA complexes were pull down, the beads were washed extensively with different buffers:

- 3x Mixed Micelle buffer [1% Triton-X-100, 0.2% SDS, 150mM NaCl, 20mM Tris-Cl pH 8.1, 5mM EDTA pH 8.0, 5.2% Sucrose, 0.02% NaN₃]
- 2x Buffer 500 [1% Triton-X-100, 0.1% deoxycholic acid, 1mM EDTA pH 8.0, 50mM HEPES pH 7.5, 0.2% NaN₃]
- 2x LiCl/detergent solution [0.5% deoxycholic acid, 1mM EDTA pH 8.0, 250mM LiCl, 0.5% NP-40, 10mM Tris-Cl pH 8.0, 0.02% NaN₃]
- 1xTE buffer [1mM EDTA, 10mM Tris-Cl pH 8.0]

Beads-Antibody-Protein-DNA complex disruption:

Then the Beads-Antibody was removed from the Protein-DNA complex using a 1% SDS+ 0.1M NaHCO₃ solution during 20 minutes of rotation at room temperature. We centrifuged gently and kept the supernatant, containing the soluble Protein-DNA complexes.

After that, the crosslinking between the Protein and the DNA was reversed by incubation with a 5M NaCl solution overnight at 65°C and shaking at 900rpm.

DNA purification:

After the Proteins were separated from the genomic DNA, the DNA was purified with a conventional DNA purification kit (PCR purification kit-Qiagen). The purified DNA was analyzed with qPCR using specific primers to detect the genome sequence of interest.

Genomic DNA quantitative PCR (qPCR):

To quantify the binding of HIF to genomic DNA specific sequences, we selected a gene that is well known as HIF-dependent gene: Carbonic anhydrase *ca9* (also termed *caIX*).

As shown in figure 2, HIF IP resulted in an enrichment of the *ca9* gene HRE DNA when we exposed the cells to hypoxia (Fig.2). This enrichment was not observed in the IgG IP used as negative control. Since the histones are associated to genomic DNA, the H3 IP was used as a positive control. In this case we observed a very good enrichment of the *ca9* gene HRE DNA in normoxia, although it was reduced in hypoxia. The transcription initiation site of *ca9* is located very close to the HRE where HIF binds to induce the gene expression, and therefore the decrease on H3 binding reflects H3

dissociation at the initiation transcription site to allow gene transcription under hypoxic conditions.

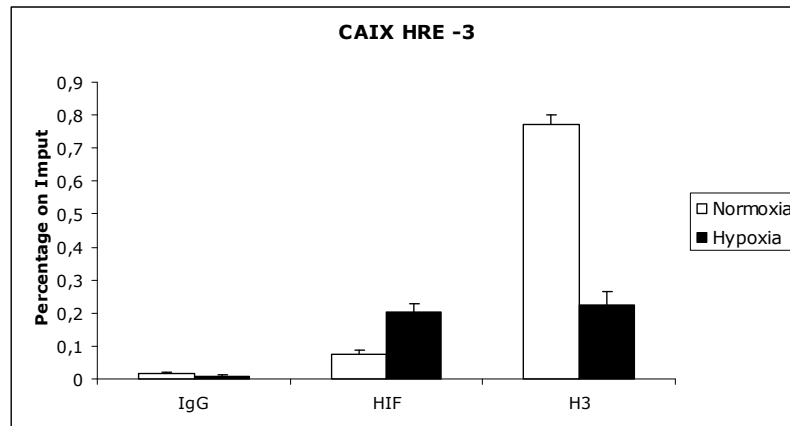


Figure 2: ChIP in HeLa cells exposed to normoxia or hypoxia for 6hs. The extracts were immunoprecipitated with IgG, anti-HIF1 α or anti-H3, and finally the enriched *ca9* HRE DNA was analyzed by qPCR.

❖ *Validation of our home-made HIF1 α antibody*

In parallel, we tested the ability of our anti HIF antibody (home-made antibody) to perform the ChIP assay. Therefore, we used the same HeLa extracts that was tested before, but using at this time IgG as negative control and the home-made anti-HIF1 α antibody for HIF IP. As it is shown in the figure 3, our antibody worked as efficiently as the commercial HIF antibody. Indeed, *ca9* HRE was largely increased in the HeLa extracts incubated in hypoxia

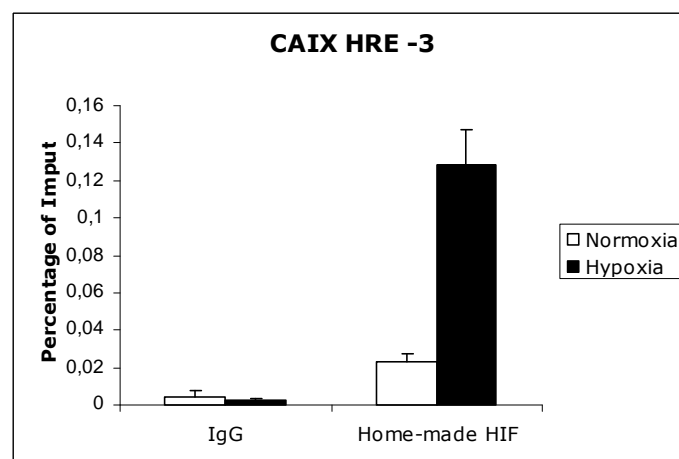


Figure 3: ChIP in HeLa cells exposed to normoxia or hypoxia for 6hs. The extracts were immunoprecipitated with IgG or our home-made anti- HIF1 α . Finally the enriched *ca9* HRE DNA was analyzed by qPCR.

❖ *ChIP in Hepatocarcinoma-derived Mouse cells (HMC)*

We also performed ChIP assays using a different cell line: hepatocarcinoma derived-mouse cells. Since we have never performed ChIP assays in these cells, we extrapolated the ChIP protocol from HeLa cells.

mRNA quantification:

However, prior to the ChIP analysis, we analyzed the expression of for tree well known HIF inducible target genes: *egln3*, *ca9* and *vegf* (Fig. 4) in our experimental conditions. We purified the mRNA and performed qPCR. We measured a great induction of *egln3* mRNA in these cells when were exposed to hypoxia, whereas *ca9* and *vegf* induction was lower compared to *egln3*.

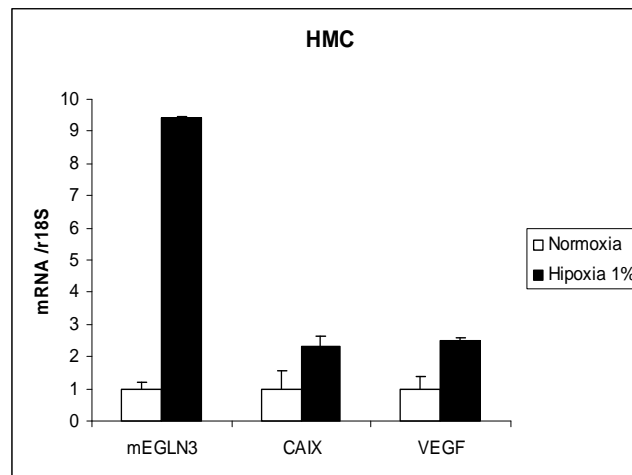


Figure 4: mRNA quantification in HMC exposed to normoxia or hypoxia for 16hs. Ribosomal 18S RNA was used to normalize.

Genomic DNA quantitative PCR (qPCR):

For the ChIP assays, we designed specific primers to amplify two different genomic DNA regions of each of the three HIF-dependent genes (*egln3*, *ca9* and *vegf*).

- HRE sequences previously reported
- sequences at positions that were 1kb distant from the HREs in the respective genes served as negative controls

Although we obtained small enrichments that showed relatively big error bars, we enriched *egln3* HRE DNA in extracts of cells incubated in hypoxia (Fig. 5A) whereas as

expected, the amplification of the 1kb genomic region distant from the HREs did not show DNA enrichment (Fig. 5B).

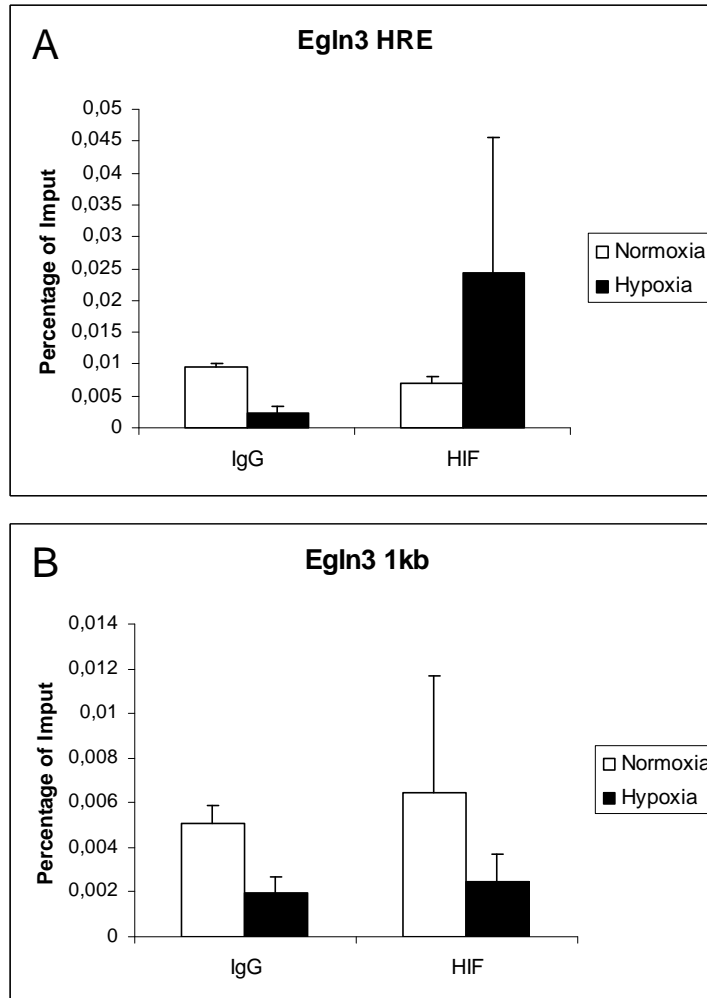


Figure 5: ChIP in HMC exposed to normoxia or hypoxia for 16hs. The extracts were immunoprecipitated with IgG or HIF1 α and then the enriched *egln3* HRE (A) or *egln3* 1kb (B) DNA was analyzed by qPCR.

We were not capable to amplify *ca9* neither *vegf*. However, these results might be more or less expected since *ca9* and *vegf* were induced only slightly in our experimental model (see Fig.4).

❖ *Final remarks*

The STSM period that I carried out in Dr Peter Staller's laboratory during two weeks was useful and constructive for my research formation. I learned to perform the chromatin immunoprecipitation assay. I also had time to gain experience in two different cell line models: HeLa cells and Hepatocarcinoma-derived mouse cells. After all of that, I will continue with the ChIP experiments in our laboratory at CIC bioGUNE where I am a PhD student under the supervision of Dr Edurne Berra. Even I still need to

optimize the assay in my lab, the experience that I received in this travel is essential for the line investigation that we are actually developing, and it will allow us to keep going with our research.