Are cartilage *SOX9* transcriptional enhancers regulated by DNA methylation and autoregulated by SOX9 protein?

Meagan Farrington (210169380), Biomedical Sciences, m.e.b.farrington2@newcastle.ac.uk, Supervisor: Dr Louise Reynard

Introduction:

Osteoarthritis (OA) is a common disorder of the joints and is the leading cause of disability in the UK[1], affecting around 10 million people[2]. OA is characterised by progressive loss of articular cartilage; however, the molecular aetiology is poorly understood.

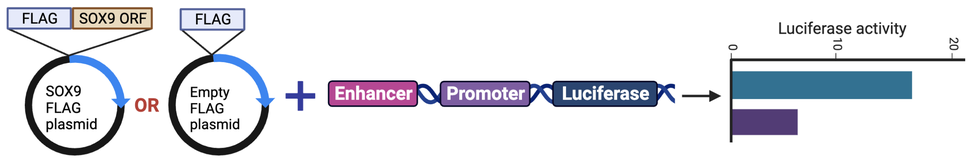
Enhancers are regions of DNA that increase how much of a gene is made. Activity of enhancers can be changed by addition of chemical groups such as methyl groups. Genome wide methylation analysis has implicated abnormal DNA methylation of cartilage transcriptional enhancers in OA.

Previous work has identified 16 enhancers of the human *SOX9* gene. The *SOX9* gene is essential in cartilage development and maintenance, making it a key gene in OA. Furthermore, mutations within *SOX9* and its regulatory elements are associated with multiple other skeletal disorders[3]. A number of these *SOX9* enhancers are hypomethylated in OA cartilage, and many also contain binding sites for SOX9 protein, suggesting SOX9 may regulate its own transcription via these enhancers. The enhancers were investigated to determine the effect of methylation and SOX9 overexpression on their activity.

Aims and methods:

Aim 1. Investigate the effect of DNA methylation on activity of *SOX9* enhancers using a luciferase reporter assay

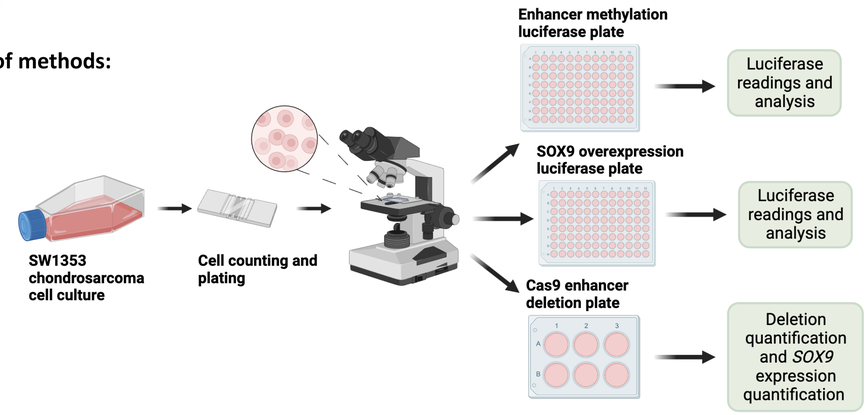
Aim 2. Investigate the effect of SOX9 protein overexpression on activity of *SOX9* enhancers



Aim 3. Produce CRISPR/Cas9 enhancer deletion cell lines and investigate the effect on *SOX9* expression

- Deleted enhancers -681b and +275, and deleted 2 enhancers together, -681a and -681b (-681a-b)
- Quantified deletion efficiencies by Qiaxcel or ImageJ analysis of PCR products, and quantified SOX9 expression using qRT-PCR

Overview of methods:



Results 1 – Luciferase reporter assays:

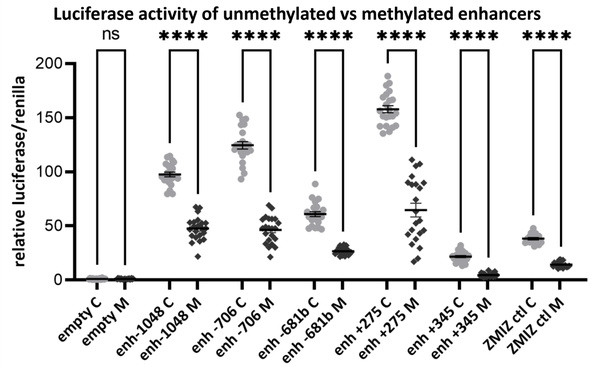


Figure 1. Luciferase reporter assay of non-methylated (C) vs methylated (M) *SOX9* enhancers. Methylation significantly decreases activity of multiple *SOX9* enhancers. (**** indicates P<0.0001, one way ANOVA followed by Šídák multiple comparison test.)

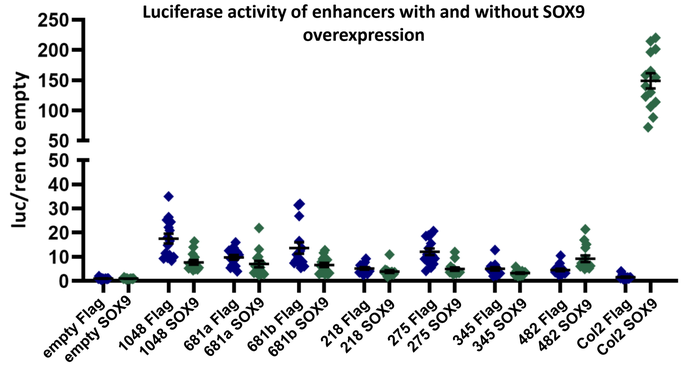


Figure 2. Luciferase reporter assay of *SOX9* enhancers with either an empty FLAG plasmid or SOX9 overexpression FLAG plasmid. SOX9 protein overexpression does not increase activity of *SOX9* enhancers and appears to decrease activity of multiple. (Only the Col2 positive control result is significant.)

Results 2 – Enhancer deletions – enhancer -681a-b as an example:

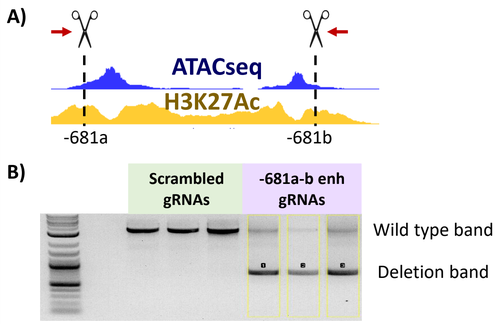


Figure 3. A) Diagram of -681a-b enhancer region. Scissors denote where gRNAs target for cutting. Arrows denote PCR primer locations. **B) PCR products visualized using gel electrophoresis.**

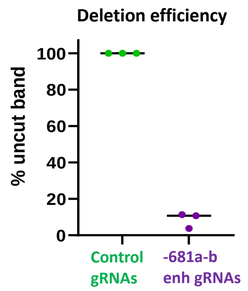


Figure 4. Deletion efficiency of -681a-b enhancer deletion. Average % of the undeleted band remaining is under 10%, indicating good deletion efficiency.

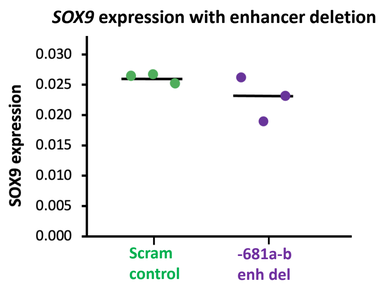


Figure 5. Effect of -681a-b enhancer deletion on *SOX9* expression. No significant difference between undeleted control (scram) and -681a-b deletion.

Conclusions and discussion:

- Methylation significantly represses activity of all 5 enhancers investigated which were hypomethylated in OA cartilage, implying these enhancers may be overactive in OA, potentially affecting cartilage homeostasis.
- Overexpression of SOX9 protein did not increase activity of *SOX9* enhancers, in fact some of the enhancers' activity decreased, which could suggest *SOX9* exhibits a tightly controlled negative feedback loop where increased SOX9 protein causes a decrease in transcription. This feedback loop could be investigated by repeating the experiment and collecting results at different time points.
- Produced new deletion cell lines with high deletion efficiency, however deletion caused no significant change in *SOX9* expression. The next steps would be to investigate the effect of CRISPR activation on these enhancers.

References:

1) Versus Arthritis. Not just 'A touch of arthritis': tackling osteoarthritis - the UK's leading cause of pain and disability. [Internet]. Versus Arthritis; 2021 [cited 2023 September 27]. 9 p. Available from: https://versusarthritis.org/media/24485/versus-arthritis-pfizer-report.pdf
2) Versus Arthritis. The State of Musculoskeletal Health 2023. [Internet]. Versus Arthritis; 2023 [cited 2023 September 27]. 6 p. Available from: https://versusarthritis.org/media/25649/versus-arthritis-state-msk-musculoskeletal-health-2023.pdf
3) Lefebvre V, Angelozzi M, Haseeb A. SOX9 in cartilage development and disease. Curr Opin Cell Biol. 2019;61:39-47.