Final Project – BIOL463 Gene Regulation in Development – Ida Vinggaard Kjeldsen

Effect of cis-regulatory element CNE14 on GLI3 expression in developing mammals

**Introduction**

Hedgehog signalling is a pathway known to regulate development of limbs and central nervous system in all bilateral animals [1]. In vertebrates, changes in gene expression in response to the signalling are mediated largely by zinc-finger transcription factors of the GLI family [2]. The main transcription factor implicated in limb development and digit patterning is GLI3, which is also expressed in nervous tissue during CNS development. Correct spatiotemporal expression of GLI3 during development (pre- and postnatally) is crucial for correct formation of limbs and craniofacial structures [2].

GLI3 expression is regulated by several cis-regulatory elements. The intronic regions of the GLI3 gene contain several conserved non-coding elements (CNEs) that are implicated in gene regulation [ref]. In 2015, Anwar *et al.* described two novel CNEs (CNE13 and CNE14) conserved in all tetrapods [2]. The individual CNEs seem to have distinct activities and tissue-specificity [2], suggesting they have differential roles in GLI3 regulation. The mechanisms of regulatory functions and tissue-specific activities remain unknown.

This project focuses on the regulatory actions of CNE14. The paper by Anwar *et al.* showed that CNE14 is capable of driving reporter gene expression in the developing pectoral fin of zebrafish, but does not induce gene expression in nervous tissue, contrary to other CNEs [2].

An *in vitro* assay in NIH3T3 cells (mouse embryonic fibroblasts) shows that CNE14 can induce reporter gene expression in a mammalian system [2], but does not address the tissue-specificity. This project sets out to investigate the regulating actions of CNE14 *in vivo* and address the tissue-specificity of CNE14-driven GLI3 expression in mammals.

**Relevance, potential impacts and importance**

Hedgehog signalling is the main regulator of limb formation and CNS development in vertebrates [1]. Dysregulation of this pathway leads to many syndromes with phenotypes including polydactyly, deformity of limbs and craniofacial structures, and can even lead to impaired brain development [3]. As GLI3 is an important modulator of this pathway, it is of great importance to characterize and understand the expression of this particular transcription factor. The emergence of two novel intragenic cis-regulatory elements only last year [2] highlights the fact that our understanding of the spatiotemporal expression of GLI3 in development is still incomplete. Characterizing this complex pattern further and expanding our knowledge acquired in zebrafish to mammals will contribute to our understanding of how Hedgehog signalling regulates proper limb and brain development in humans.

Investigating the regulatory mechanisms of CNE14, especially in regard to tissue-specificity, will also contribute to our knowledge on cis-regulatory elements in general and may provide important information that can be applied in parallel studies of other gene regulation patterns.

**Hypothesis**

My hypothesis is largely based on the two assays carried out by Anwar *et al*. in 2015 [2]. The transgenic zebrafish assay shows that CNE14 is capable of promoting reporter gene expression in the developing limb buds of zebrafish [2], suggesting that CNE14 is important for limb-specific GLI3 expression. The *in vitro* reporter assay in NIH3T3 cells revealed reporter gene expression driven by CNE14 [2], indicating that CNE14 is also an active regulatory element in mammals. This leads to the hypothesis that CNE14 is essential for normal GLI3 expression patterns in limbs of developing mammals.

The conservation of CNE14 in all tetrapods supports the hypothesis that it has a central function in regulation. Additionally, comparative genomics studies predict several putative transcription factor binding sites within the region, further supporting a regulatory role [2].

**Experimental plan and predictions**

The experiment to test the hypothesis is a knockout assay in a mouse and gene expression analysis of the embryo. The knock out mouse will carry a deletion within intron 3 of GLI3, removing CNE14. Observing the effects on GLI3 expression patterns will allow us to asses the importance of CNE14.

Model system

A mouse is an ideal model system for this project. Being a mammal, the genome and gene regulation resembles humans, which makes it probable that results obtained in mice may apply to humans as well. The relatively short development time makes it possible to create a knockout mouse for research purposes.

Creating a knockout mouse

Mouse embryonic stem cells are engineered to contain a deletion of CNE14 in the GLI3 intron. This is achieved by taking advantage of the cells’ pathway for homologous recombination. The template DNA will contain the desired deletion in GLI3 and an insertion of a drug resistance gene, flanked by regions homologous to the genome around the GLI gene. The cells are transfected with the construct by electroporation (or microinjection/use of transfecting agent?). The drug resistance gene allows positive selection of cells that have integrated the construct [4]. This knockout can be carried out with a higher efficiency by genomic editing with CRISPR/Cas9. However, I have chosen to include the other protocol, as this is the procedure of most labs that do not have access to CRISPR/Cas9 technology.

The modified ES cells are sequenced to confirm successful transfection and to ensure no frameshifts or nonsense mutations has been introduced.

The ES cells are injected into mouse blastocysts that are implanted in the uterus of a foster mother. The developed mice will be mosaics – only part of their cells will carry the mutation. The chimeric mice are mated and their offspring will be either wild type or heterozygous for the mutation, depending on whether the mutations was present in the germ cells. The offspring is genotyped to check the presence of the mutation.

The heterozygous mice are mated, and the embryos are tested for homozygosity by sequencing when extracted.

Gene expression analysis

Analysis of GLI3 gene expression in CNE14 mutants and wild type mice are done by **whole mount in situ hybridization** followed by **histochemical detection** of GLI3. The embryos are extracted at E10, which is late enough in development to allow extraction and histochemical detection. They are treated (see [5]) and incubated overnight in pre-hybridization solution with a GLI3 mRNA probe, created by PCR. The embryos are washed with blocking solution to prevent unspecific antibody binding and subsequently incubated with antibodies. After washing, the embryos are incubated with dye (staining the antibodies?) and tissue-specific expression patterns are visualized [5]. This procedure is done in knockout embryos and wild type embryos for comparison.

Predictions

If the hypothesis is correct, the knockout mouse embryos will show altered - perhaps even abrogated - GLI3 expression patterns in developing limbs compared to wild type. The GLI3 expression in CNS will likely not be affected.

**Possible results**

A possible set of results could show altered GLI3 expression patterns in limbs and/or CNS. From this we can conclude that CNE14 is involved in regulating expression of GLI3 and necessary for normal (wild type) expression patterns. These results would fit in with the current literature describing a complex regulatory pattern with many components involved.

Alternatively, we could observe no GLI3 expression in the limbs of the mouse embryo, but normal expression in CNS. This would likely be accompanied by a phenotype of polydactyly or underdeveloped limbs. From this you can conclude that CNE14 is necessary for normal GLI3 expression in developing limbs, and infer that dysregulation of GLI3 due to a CNE14 deletion leads to the observed phenotype.

Another possibility is that no GLI3 expression is observed at all. This would show that CNE14 is necessary for GLI3 expression in all tissues. Given that GLI3 is necessary for limb and CNS development, the embryo might exhibit a severe phenotype or might not develop to the stage of extraction.

It is also possible that we will see no changes in gene expression whatsoever. This will allow us to conclude that CNE14 is not necessary for GLI3 expression patterns. If CNE14 is indeed involved in the regulation, then we can infer that other mechanisms exist to make up for the deletion and restore normal expression.

Assuming the predictions are correct and the results show altered GLI3 expression in limbs, future experiments could focus on revealing how CNE14 regulation is mediated. CNE14 contains many putative binding sites for known proteins, mostly transcription factors, and assessing whether these proteins bind to CNE14 *in vitro* and later *in vivo* would shed some light on the molecular mechanism.

**Summary**

Some sort of illustration

**References**

[1] J. Briscoe, “The mechanisms of Hedgehog signalling and its roles in development and disease,” *Nature*, vol. 14, no. July, pp. 416–429, 2013.

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