Investigation and manipulation of methylation status of transiently transfected plasmid DNA in the developing retina

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Plasmid DNA has many applications in scientific research, including as a delivery method for gene therapy, as a reporter system to mark specific cell types in complex tissues, and as a vehicle for investigating transcription factor (TF) control of *cis*-regulatory elements (CREs). While plasmids are extremely useful tools, their ability (or lack thereof) to recapitulate the epigenetic modifications present in the genome is not often addressed. It is thus important to consider the effect that this loss of information may have on interpretation of results from transient transfection assays. Additionally, introducing such modifications into plasmids prior to their transfection could allow for a more targeted investigation of these modifications, whose effects are often difficult to isolate in the complex genomic environment.

One example of an epigenetic modification is DNA methylation, specifically methylation of the C5 position on cytosine nucleotides that are followed by a guanine (denoted as CpG sites). The methylation status of CpG dinucleotides in CREs has been established to affect the expression of downstream genes. Thus, CREs lose important regulatory information if their methylation status is not recapitulated in the context of a reporter plasmid. The binding affinity of some TF proteins have been identified as sensitive to the methylation status of the CpG within their DNA recognition motif, including the TF ONECUT1 (OC1); it can bind to its cognate motif containing either a CpA or a CpG dinucleotide with relatively equal affinities *in vitro*, but this binding is methylation sensitive.[1,2] OC1 serves an important role in development of the retina, and binds to an element termed ThrbCRM1, which contains a highly conserved CpA dinucleotide.[3] Mutational analysis of this sequence in the context of a reporter plasmid has demonstrated no detectable loss of activity in mutating the CpA to a CpG; this indicates that either the transiently transfected plasmid does not obtain CpG methylation (mCpG), or that in this context OC1 can still effectively bind to mCpG. Initial experiments indicate that transiently transfected plasmids into the retina do not acquire mCpG. Not only is this important for interpretation of results from reporter assays, but this affords a unique opportunity to gain insights into how mCpG can affect the binding affinity of TFs like OC1. *In vitro* methylation of the reporter plasmid prior to transfection demonstrated a decrease in activity, which is more pronounced in the CpG-containing OC1 binding motif. To refine the results of this experiment, a nickase-based strand replacement method was employed to investigate the effect of mCpG in the binding motif for OC1, rather than across the entirety of the plasmid. This method also allows generation of a hemi-methylated plasmid, which provides functional insights into the structural basis for the decrease in TF binding affinity caused by methylation.

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