Lighting Up Embryos

Human Developmental Biology Initiative

Newcastle University

3 GOOD HEALTH AND WELL-BEING

1 Testing a Novel CRISPR-Mediated Gene Integration Method for Investigating Reproductive Health

2 Shld1 System for Modulating Protein Expression

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Aims:

- To understand, recreate and implement novel genome editing technology, 'PASTE', to integrate green fluorescent protein (GFP) into the genome of developing mammalian embryos
- To fluorescently tag Sox2 transcription factor to visualize cellular processes occurring during preimplantation development, from fertilization to implantation

Introduction:

An estimated 8 million children have been conceived globally using assisted reproductive technologies (ART), such as IVF, to treat infertility. Developing tools for improving our knowledge of the earliest stages of development will allow us to assess new fertility treatments and fulfil our responsibilities to the health of further generations of ART-conceived children.

What is 'PASTE'?

- Programmable Addition via Site-Specific Targeting Elements -

A new genome editing tool that can programme integration of large genes (36,000 base pairs) into targeted genomic loci[1]. This technology does not induce double-strand breaks (DSBs) in DNA, which is required by alternative gene editing methods, such as CRISPR-Cas9.

Chromosome loss in human embryos following induction of DSBs has been reported, hence the importance of exploring DSB-independent approaches for germline genome editing[2].

How does 'PASTE' work?

① Prime editing, a precise gene editing method, inserts an 'AttB' site at a desired location in the genome, via reverse transcription and flap repair

② 'AttB' landing site acts as a beacon for insertion of the 'AttP' site, along with the gene of interest at a specific genomic loci

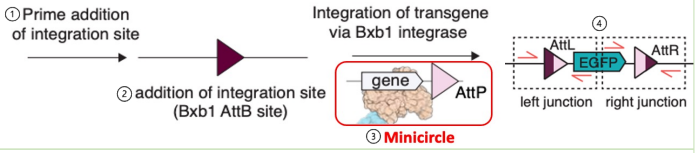

Figure 1. Schematic of programmable gene insertion with PASTE.

③ Deliver minicircles, circular double-stranded DNA template, that contain the gene of interest (e.g. GFP) and an 'AttP' site

④ Bxb1 serine integrase – an enzyme that binds to AttP and AttB sites, the sites come together, and site-specific recombination generates two new sites: AttL and AttR

Design:

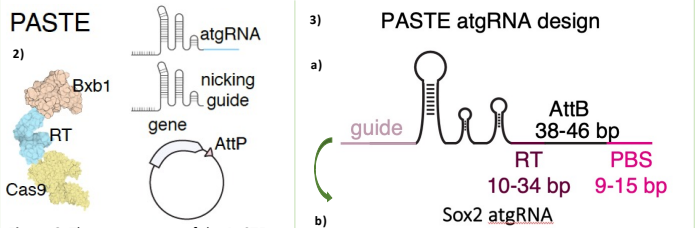

Figure 2. The components of the PASTE system. PASTE expression vector: Fusing of Bxb1 site-specific serine integrase, reverse transcriptase (RT) and Cas9 nickase (not nuclease so no DSBs of DNA). The atgRNA, nicking guide (for stimulating repair of DNA strand) and the minicircle DNA encoding gene of interest and AttP site. We designed and made these components to tag the Sox2 gene (lineage marker for epiblast specification) with GFP.

Figure 3. (a) Diagram showing the general design of atgRNA for optimal integration of the AttB site into the genome: the PBS, RT, and AttB lengths can alter the efficiency of AttB insertion. ***(b)*** SnapGene software enabled the design of atgRNA for integrating the AttB site at the N-terminus of the Sox2 gene. We made a synthetic DNA fragment, that was PCR amplified for in vitro transcription of the atgRNA. ***(c)*** After prime editing, the AttB site is integrated into the genome at a specific location, determined by the atgRNA. ***(d)*** The minicircles lead to this final product of the genome with GFP integrated, so fluorescent protein can be expressed with the Sox2 gene and visualized.

Strategy:

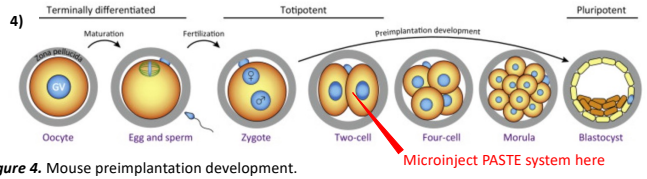

Figure 4. Mouse preimplantation development.

Eggs were harvested from the ovaries of Cd1 mice, zygotes identified (those that had been fertilized) and incubated until they developed to two-cell stage. mRNA of the 'PASTE' system components were delivered to the cells via microinjections and live-imaging was carried out using fluorescence microscopy. Injection of mRNA at mouse 2-cell stage has improved the integration efficiency of fluorescent protein due to a prolonged G2 phase[3].

Conclusion:

After analyzing live imaging of the embryos three days post microinjection of 'PASTE', the system had not worked as integration of GFP was not successful. 'PASTE' should be tested again upon improving the yield and purity of the minicircles containing the GFP and re-evaluating the atgRNA design for Sox2. This technology has the potential to transform germline genome editing and advance our knowledge of early embryo development, understand major causes of infertility and extend the scope of reproductive technologies to prevent transmission of disease to improve the health of future generations.

Aims:

- Evaluate the use of a Shld1 responsive destabilizing domain for modulating protein expression in mouse eggs [4].

Introduction:

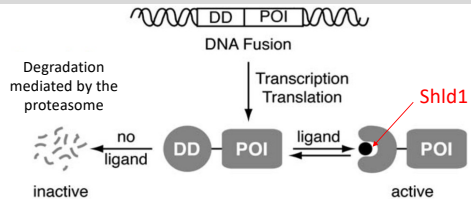

Figure 5. General method to conditionally control protein stability.

Diagram showing a destablizing domain (DD), a mutant form of a human protein, which is unstable and rapidly degraded when expressed in mammalian cells. Genetic fusion of DD to a protein of interest (mScarlet-I & mNeonGreen (fluorescent proteins)) leads to the fused protein also being unstable and therefore degraded.

The Shld1 compound is a synthetic ligand that binds to DD and shields the protein from degradation. The fused protein can now be expressed by the cell. This system allows precise regulation of protein stability in a cell.

Results:

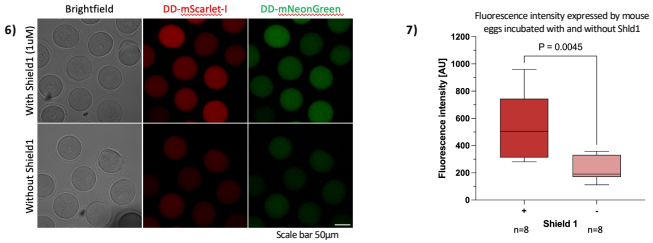

Figure 6. Immunofluorescent staining images showing the expression of mScarlet-I and mNeonGreen protein in Cd1 mouse eggs, 5 hours post microinjection of synthesized mRNA, in the presence (top) and absence (bottom) of a chemical containing the Shld1 ligand.

Figure 7. Graph displaying the significant difference in measured fluorescence intensity expressed by the eggs between the Shld1 +/- groups.

Conclusion:

Significantly more protein was expressed when the Shld1 ligand was present, as expected, but fluorescent protein was produced even without Shld1, which was unexpected. The translation rate of the protein appeared to overcome the degradation rate, despite no ligand present. This experiment needs to be optimized and repeated to confirm the efficacy of fusing a Shld1 responsive destabilizing domain to control protein stability in this cell type. This method is unique as it allows reversible control of protein function by addition/removal of Shld1 ligand. The long-term goal would be to combine Shld1 with 'PASTE' in order to 'switch on and off' the gene editing function at specific points in embryo development.

References:

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2. Zuccaro MV, Xu J, Mitchell C, Marin D, Zimmerman R, Rana B, et al. Allele-Specific Chromosome Removal after Cas9 Cleavage in Human Embryos. Cell. 2020; 183(6):1650-1664.
3. Gu B, Posfai E, Rossant J. Efficient generation of targeted large insertions by microinjection into two-cell-stage mouse embryos. Nat Biotechnology. 2018; 36: 632-637.
4. Banaszynski L, Chen L, Maynard-Smith L, et al. A Rapid, Reversible, and Tunable Method to Regulate Protein Function in Living Cells Using Synthetic Small Molecules. Cell. 2006; 126(5):995-1004.