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Morpholino-Mediated Gene Knockdown in Mammalian Organ Culture

Alda Tufro

Abstract

We examined the role of semaphorin3a in ureteric bud (UB) branching morphogenesis using mouse metanephric organ culture [Tufro et al. (*Mech Dev* 125:558–568, 2008)]. In vitro UB injection of *Sema3a* antisense morpholino resulted in increased branching morphogenesis. Cellular and tissue uptake of oligonucleotides was facilitated by a peptide-mediated method. Our findings were validated by in vitro translation and in *Sema3a* null mice. This chapter describes a method to perfuse the UB lumen with fluorescein-labeled oligonucleotides bound to a peptide carrier.

Keywords

Metanephric organ culture; Ureteric bud perfusion; Peptide–morpholino-mediated gene knockdown

1. Introduction

Branching morphogenesis of the ureteric bud (UB) is essential for kidney development (1). Mutations that alter ureteric bud branching result in renal agenesis, hypoplasia, multiple or dysplastic kidneys (1, 2). UB branching requires reciprocal signals to and from the surrounding mesenchyme and stroma (1, 2). Given the multitude of non-cell autonomous signals involved, and the very specific time and spatial constraints of UB branching development, it is necessary to timely examine UB branching in metanephric organ culture or embryonic kidneys.

Gene knockdown through RNA interference or translation inhibition are useful approaches to elucidate gene function (3). However, poor cellular uptake of nucleic acids and degradation by nucleases are major limitations of these technologies (3–5). Viral and nonviral strategies have been used to overcome this in cell culture and in vivo. Lipid-mediated nucleic acid transfection can be achieved in cell lines, but is not efficient in vivo or in organ culture. Peptide-mediated cell uptake of macromolecules, including oligonucleotides, proteins, and peptides has also been reported (5, 6). Cell-penetrating peptides are either covalently coupled or form noncovalent complexes with plasmid DNA or oligonucleotides. We used a 27 residues translocator peptide, called MPG, consisting of an internalization domain derived from the fusion sequence of HIV protein gp41, a nuclear localization domain derived from the NLS of SV-40 large T antigen, and a spacer domain in

between them (6). This peptide is stable at physiologic pH and in the presence of serum, and the peptide–oligonucleotide complex dissociates rapidly after it crosses the cell membrane (7). Morpholinos are oligonucleotides modified by nonionic phosphorodiamidate linking morpholine rings that replace deoxyribose moieties, to prevent degradation by nucleases (3). Antisense morpholinos are widely used to achieve gene knockdown by injection into *Xenopus* oocytes and zebrafish blastocysts (3). Their use in mammalian cells has been limited by poor cell uptake.

We developed a method to achieve *sema3a* gene knockdown in mouse metanephric organ culture using a cell-penetrating peptide–morpholino complex microinjected into the UB lumen in vitro (8). This method may also be used to deliver plasmid cDNA into the UB. This chapter describes the protocols used for peptide–morpholino-mediated knockdown and mouse UB microperfusion.

2. Materials

2.1. Organ Culture

1. Defined serum-free organ culture medium is DMEM/F12 without phenol red, supplemented with 10 mM Hepes, 5 mg/ml insulin, 5 mg/ml transferrin, 2.8 nM selenium (Gibco, #41400), 25 ng/ml PGE1 (Sigma, #P7527), 32 pg/ml T3 (Sigma, T5516), 50 U/ml penicillin, and 50 U/ml mycostatin (see Note 1).
2. Organ culture filters are Millicell-CM Biopore PTFE (Millipore).
3. Organ culture dishes and glass bottom culture dishes (MatTek Corp., #P35GC-1.5-10-C).

2.2. Morpholino Oligonucleotides and Peptide Carrier

1. Fluorescein-labeled *Sema3a* antisense morpholino (Genetools, LLC) is 5'-aggcaatcccagtgaaccagcccat-3'-carboxyfluorescein. Control morpholinos are 5 bp-mismatched morpholinos.
2. Translocator peptide MPG (GALFLGFLGAAGSTMGAWSQPKSKRKV) (6).

2.3. Microperfusion System

1. Injectman NI2 micromanipulator/Femtojet semiautomated microperfusion system (Eppendorf).
2. Glass microcapillary needles (Femtotips II, Eppendorf).

2.4. Imaging

1. Phase microscope (IX81, Olympus).
2. Confocal microscope (Fluoview300, Olympus).

3. Methods

3.1. Metanephric Organ Culture

1. Kidneys are excised from E11.5 mouse embryos by microdissection (see Note 2).
2. Left and right kidneys are placed on separate organ culture filters inside glass bottom culture dishes (see Note 3).

3.2. Preparation of Peptide–Morpholino Complexes

1. Preparation of MPG solution:
 - a. Remove MPG vial from freezer and leave closed at room temperature for 30 min.
 - b. Resuspend in sterile water at 1 mg/ml (0.35 M), vortex briefly.
 - c. Aliquot to avoid repeated freeze–thaw cycles. Stock solution is kept at -20°C or -80°C .
2. Preparation of morpholino solutions:
 - a. Oligos are delivered as sterile, salt-free, lyophilized solid in glass vials.
 - b. Make stock solution to 1 mM in distilled water (see Note 4).
 - c. Keep stock solutions at -20°C .
3. Preparation of peptide–morpholino complexes:
 1. Mix morpholinos (1 nmol/ μl) with MPG peptide at 1:5–1:10 molar ratio.
 2. Incubate at 37°C for 30 min.
 3. Microinject peptide–morpholino complexes (1 nmol/ μl), or add to organ culture medium to final morpholino concentration of 10 μM (see Note 5).

3.3. Ureteric Bud Injection

1. Place organ culture filter with freshly microdissected E11.5 kidney on a glass bottom culture dish with culture media.
2. Place the dish on the microinjection workstation mounted on the microscope and take picture of explant ($\times 10$) on phase.
3. Program Femtojet parameters for injection time 2–30 s, injection pressure 50–200 hPa.
4. Fill microcapillary with oligonucleotide–peptide mix in micro-manipulator holder and place at 30 – 60° angle in desired location.
5. Program the injection level (Z-axis) on the Injectman, or place it on “standby” to inject manually.
6. Activate the joystick key to inject (see Note 6).

7. Take confocal picture of explant ($\times 10$) post injection to visualize fluorescein in UB lumen.

3.4. Metanephric Organ Culture

1. Place filters with injected explant in organ culture dish in incubator at 37°C, 5% CO₂.
2. Change media daily and monitor branching by phase microscopy live.
3. After 48–72 h fix explants in 10% buffered formalin for lectin or immunostaining, or harvest explants in lysis buffer for RNA or protein analysis.

4. Notes

1. The organ culture chambers should be filled with enough defined medium to bathe but not submerge the embryonic kidneys, and distilled water used to fill the outside compartment to prevent explant drying.
2. Care should be taken during microdissection of embryonic kidneys to remove the entire length of the UB intact, because if it is cut too close to the metanephros the perfusate back-leaks immediately.
3. To control for variability among embryos and nontarget effects, microinject antisense morpholino–peptide complex into one UB and the corresponding mismatched morpholino–peptide complex to the contralateral one.
4. Morpholinos can be damaged by DEPC.
5. Additional controls should include MPG peptide alone into one UB, and mismatched morpholino–peptide complex into the contralateral UB, as well as antisense and mismatched morpholinos alone.
6. If the injection “missed” the UB lumen, the microcapillary can be placed at different position and procedure tested again, multiple punctures may cause back-leak and poor perfusion.

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