Abstract
Oligonucleotide therapeutics (ONTs) encompass classes of medicines that selectively target and potentially ameliorate previously untreatable and often rare diseases. Several unique classes of ONTs provide versatility, enabling direct modulation of gene expression by virtue of Watson-Crick base pairing or modulation of cell signaling through structural mimicry or interference with protein-receptor interactions. Due to a lack of suitable in vitro models capable of recapitulating or predicting in vivo effects of ONTs, their discovery and optimization has relied heavily on animal studies for predicting efficacy and safety in humans. Since ONTs often lack cross-species activity, animal models with genetic humanization and/or species-specific surrogate ONTs are often required. Human microphysiological systems (MPS) offer an opportunity to reduce the use of animals and may enable evaluation of drug mechanisms, optimization of cell and tissue targeting ligands or delivery vehicles, and characterization of pharmacokinetics (PK), pharmacodynamics (PD), and safety of candidate ONTs. The lack of published examples for MPS applications with ONT demonstrates the need for a focused effort to characterize and build confidence in their utility. The goals of this review are to summarize the current landscape of ONTs and highlight potential opportunities and challenges for application of MPS during ONT discovery and development. In addition, this review aims to raise awareness with ONT drug developers and regulatory authorities on the potential impact of MPS with respect to characterizing pharmacology, ADME, and toxicity and to educate MPS platform developers on unique design attributes needed to fully appreciate MPS advantages in ONT development.

1 Introduction
Microphysiological systems (MPS) and complex in vitro models are gaining traction in drug discovery and development but have primarily been evaluated using small molecules with a focus on toxicological and pharmacological endpoints. The adoption of MPS offers a promising avenue to reduce animal use, improve in vitro-to-in vivo translation including PK/PD/toxicity correlation, and provide mechanistic evidence of species concordance (or lack thereof). While MPS have demonstrated utility in these areas with small molecules and biologics (Peterson et al., 2020), wider adoption of MPS to drug development warrants validation of their use for other modalities including ONTs. However, there is currently limited published evidence of MPS being utilized to study the disposition, metabolism, pharmacology, and toxicity profiles of ONTs. Key features of MPS, including long-term viability and physiologically relevant expression of functional enzymes, receptors,
<table>
<thead>
<tr>
<th>Trade name</th>
<th>Generic name</th>
<th>Year</th>
<th>FDA approved</th>
<th>Chemistry, delivery</th>
<th>Target</th>
<th>Route, freq.</th>
<th>Indication(s)</th>
<th>Bioanalytical method</th>
<th>Preclinical pharmacology model(s) and notable findings</th>
<th>Human clinical trials – serious TEAEs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitavene</td>
<td>Fomiviren</td>
<td>1998</td>
<td>PS-ASO</td>
<td>CMV/IE2</td>
<td>i.v.t</td>
<td>Q2Wx2, Q4W</td>
<td>CMV retinitis</td>
<td>Unknown (LLOQ 25 nM in vitreous, unknown in plasma)</td>
<td>Human: <em>in vitro</em> antiviral activity</td>
<td>Rabbit: ocular inflammation (cyclois) NHP: no ocular findings Mouse (i.v.): stromal hyperplasia and bone marrow megakaryocyte depletion, chronic liver inflammation</td>
</tr>
</tbody>
</table>
| Macugen   | Pegaptanib  | 2004 | PEG-aptamer   | VEGF<sub>165</sub> | i.v.t., Q6W | Neovascular AMD | Human: *in vitro* gene expression, function | Dual, double hybridization assay HPLC (plasma) | Human: *in vitro* gene expression, function | NHPs, rabbits, and dogs: only injection-related findings 
Rats (i.v.): nephropathy and lymphoid depletion in spleen, vacuolated macrophages in multiple tissues | Endophthalmitis | Intracocular pressure Anaphylaxis (incl. angioedema) |
| Kynamro   | Eptinez     | 2013 | PS-ASO       | ApoB               | s.c., QW | Familial hypercholesterolemia | Human, NHP: *in vitro* gene expression | Hybridization-based ELISA plasma (0.23–30.4 ng/mL); CGE-UV urine (0.076–1.6 µg/mL) | Human, NHP: *in vitro* gene expression | NHP: kidney tox., thrombocytopenia 
Mice: liver toxicity 
Rats: liver and kidney tox., prolonged APTT 
All: inflammation, cardiovascular effects | Hepatotoxicity (↑ ALT) 
Hepatic steatosis |
| Defitelio | Defibrotide Sodium | 2016 | d | d | i.v., Q6H | Hepatic VOD w/renal or pulmonary dysfunction after HSCT | Human: *in vitro* gene expression, function | UPLC/UV: fluorometric ssDNA assay (plasma, dialysate; LLOQ 0.2 µg/mL) | Human: *in vitro* gene expression, function | Rat: liver and kidney toxicity, lymphoid organ toxicity 
Rats and dogs: prolonged APTT and PT | Hyper-sensitivity (Anaphylaxis) Hemorrhage |
<table>
<thead>
<tr>
<th>Therapy</th>
<th>Nusinersen</th>
<th>2016</th>
<th>PS-SSO</th>
<th>SMN2 pre-mRNA (ISS-N1)</th>
<th>i.t., Q2Wx3, Q4Wx1, Q4M</th>
<th>Spinal muscular atrophy</th>
<th>hybridization ELISA or ECL assay (CSF and plasma; linearly in plasma only)</th>
<th>Human: in vitro gene expression</th>
<th>NHP: in vivo gene expression Mouse (wt, mdx): in vivo gene &amp; protein expression (surrogate)</th>
<th>Rat: ↓ bone mineral density</th>
</tr>
</thead>
<tbody>
<tr>
<td>ONPATTRO</td>
<td>Patisiran</td>
<td>2018</td>
<td>PS-ASO</td>
<td>TTR</td>
<td>s.c., QW</td>
<td>hATTR polyneuropathy</td>
<td>hybridization ELISA (1 – 200 ng/mL in plasma)</td>
<td>Human: in vitro gene expression Mouse (tg), NHP: in vitro and in vivo gene &amp; protein expression</td>
<td>NHP: thrombocytopenia, vasculitis Mouse: no notable findings Rat: renal toxicity</td>
<td>Thrombocytopenia Renal tox. (glomerulonephritis)</td>
</tr>
<tr>
<td>Vyondys 53</td>
<td>Golodirsen</td>
<td>2019</td>
<td>PMO-ASO</td>
<td>DMD pre-mRNA exon 53</td>
<td>i.v., QW</td>
<td>Duchenne muscular dystrophy</td>
<td>LC fluorescent; 1-250 ng/mL plasma (30 µL injection)</td>
<td>Human: in vitro gene expression NHP, mouse (tg): in vivo gene &amp; protein expression</td>
<td>Rat and NHP: hepatotoxicity; ↓ vitamin A Rats: ↓repro effects, injection site rxn</td>
<td>Infusion rxn ↓ vitamin A</td>
</tr>
</tbody>
</table>

**NHP:** acute neurological effects; neuronal vacuolation, hippocampal necrosis

**Mouse (s.c.):** liver and kidney toxicity, lymphoid organ toxicity

**Rat:** renal toxicity

**Thrombocytopenia:** Renal tox. (glomerulonephritis) Stroke and carotid dissection Inflammation/immune effects Liver effects (↑ ALT) Hypersensitivity ↓ vitamin A

**Hyper-sensitivity**
cies cross-reactivity, poor in vitro-to-in vivo correlation using conventional models, and reliance on delivery systems to enable productive uptake into many cell types. While often considered a “new” or “emerging” modality, regulatory approval of the first ONT, fomivirsen, was granted by the United States Food and Drug Administration (FDA) in 1998. Since then, ONTs have gained marketing authorization globally. To date, 12 ONTs spanning vari-

<table>
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<tr>
<th>Trade name</th>
<th>Generic name</th>
<th>Year FDA approved</th>
<th>Chemistry, delivery</th>
<th>Target$^b$</th>
<th>Route, freq.</th>
<th>Indication(s)</th>
<th>Bioanalytical method</th>
<th>Preclinical pharmacology model(s)</th>
<th>Preclinical safety model(s) and notable findings$^c$</th>
<th>Human clinical trials – serious TEAEs</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIVLAARI</td>
<td>Givosiran</td>
<td>2019</td>
<td>GalNAc-siRNA</td>
<td>ALAS1</td>
<td>s.c., QM</td>
<td>Acute hepatic porphyria</td>
<td>LC-MS/MS of parent and 3N-1, LLOQ: 10 ng/mL plasma, 50 ng/mL urine</td>
<td>Human: NHP: in vivo gene expression NHP: in vivo gene expression Mouse, rat (wt; AIP model): in vivo function, gene expression</td>
<td>NHP: hepatotoxicity, ↑creatinine 1♀ Rat: hepatotoxicity; muscle degeneration at injection site</td>
<td>Anaphylaxis Hepatotoxicity (↑ALT) Renal toxicity (↑creatinine) Injection site rxn</td>
</tr>
<tr>
<td>Viltepso</td>
<td>Viltolarsen</td>
<td>2020</td>
<td>PMO-ASO</td>
<td>DMD premRNA exon 53</td>
<td>i.v., QW</td>
<td>Duchenne muscular Dystrophy</td>
<td>LC-MS/MS; LLOQ 20 ng/mL plasma</td>
<td>Human: in vivo gene &amp; protein expression NHP: in vivo gene expression Mouse (wt, tg): in vivo gene expression (surrogate)</td>
<td>Mouse and NHP: renal toxicity</td>
<td>None reported</td>
</tr>
<tr>
<td>OXLUMO</td>
<td>Lumasiran</td>
<td>2020</td>
<td>GalNAc-siRNA</td>
<td>HAO1</td>
<td>s.c., QM load, QM or Q3M</td>
<td>Primary hyperoxaluria type 1</td>
<td>LC-TOF-MS; LLOQ 10 ng/mL in plasma (100 µL injection)</td>
<td>NHP: in vivo and in vitro gene expression Mouse (wt, tg): in vivo gene expression, glycolate/oxalate Rats (wt, PH1): in vivo gene expression, glycolate/oxalate</td>
<td>Rats, NHP: no adverse effects reported in chronic studies</td>
<td>None reported</td>
</tr>
</tbody>
</table>

$^a$ Data from drug approval documents publicly available at https://www.accessdata.fda.gov/scripts/cder/daf/; $^b$ Gene and protein names are abbreviated per HGNC guidelines; $^c$ Unless otherwise specified, route of administration in preclinical species was the same as the clinical route; $^d$ Defitelio consists of a polydisperse mixture that is derived from porcine intestinal tissue and exhibits pro-fibrinolytic and anti-thrombotic properties.

and pharmacological targets make them attractive tools for nonclinical characterization of ONT.

### 1.1 Oligonucleotide therapeutics (ONTs)

Oligonucleotide therapeutics (ONTs) constitute several classes of nucleotide-based modalities with uniquely long tissue half-lives, prolonged duration of pharmacodynamic (PD) effect, limited species cross-reactivity, poor in vitro-to-in vivo correlation using conventional models, and reliance on delivery systems to enable productive uptake into many cell types. While often considered a “new” or “emerging” modality, regulatory approval of the first ONT, fomivirsen, was granted by the United States Food and Drug Administration (FDA) in 1998. Since then, ONTs have gained marketing authorization globally. To date, 12 ONTs spanning vari-
Each mechanism of action, resulting in several ONT classes, each with specific properties (Fig. 1). The most common ONT classes evaluated in clinical studies to date are antisense oligonucleotides (ASOs) and short interfering RNAs (siRNAs). ASOs are single-stranded and have a length of 12-20 nucleotides, often with a phosphorothioate (PS) backbone and 2’ ribose modifications such as methoxyethyl (MOE), locked nucleic acid (LNA), or 2’OMe. Multiple ONTs have been approved by the FDA (Tab. 1). Additionally, multiple ONTs are being explored in early- and late-stage clinical development (Tab. 2).

Depending on the intended mechanism of action, ONTs may range from 10 to 100 nucleotides in length and often contain chemical modifications of the backbone and ribose sugar (Fig. 1). The design and chemical modifications of an ONT are tailored for each mechanism of action, resulting in several ONT classes, each with specific properties (Fig. 1). The most common ONT classes evaluated in clinical studies to date are antisense oligonucleotides (ASOs) and short interfering RNAs (siRNAs). ASOs are single-stranded and have a length of 12-20 nucleotides, often with a phosphorothioate (PS) backbone and 2’ ribose modifications such as methoxyethyl (MOE), locked nucleic acid (LNA), or...
ble-stranded ONTs where each strand is approximately 20-24 nucleotides in length and may contain PS linkages and modifications to the 2' ribose that confer metabolic stability. These ONT classes rely on loading of the antisense strand into the RNA-induced silencing complex (RISC) for activity. The design of siRNA results in catalytic cleavage and subsequent degradation of the target transcript, whereas miRNA-mimics bind to endogenous constrained ethyl (cEt). These modifications improve drug properties such as metabolic stability, tissue uptake, and target binding affinity. Antisense oligonucleotides include gapmers, which trigger RNase H-mediated degradation of target transcripts, and steric blocking ASOs, which can modulate splicing events or inhibit activity of microRNA (miRNA) (Crooke et al., 2021). Short interfering RNAs and miRNA-mimics are two classes of double-stranded ONTs where each strand is approximately 20-24 nucleotides in length and may contain PS linkages and modifications to the 2' ribose that confer metabolic stability. These ONT classes rely on loading of the antisense strand into the RNA-induced silencing complex (RISC) for activity. The design of siRNA results in catalytic cleavage and subsequent degradation of the target transcript, whereas miRNA-mimics bind to endogenous

Tab. 2: Overview of ONTs in late-stage clinical development and their current status

<table>
<thead>
<tr>
<th>Drug</th>
<th>Modality</th>
<th>Target</th>
<th>Indication</th>
<th>Phase</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oblimersen</td>
<td>ASO</td>
<td>BCL2</td>
<td>Lung cancer</td>
<td>2/3</td>
<td></td>
</tr>
<tr>
<td>Sepofarsen</td>
<td>SSO⁶</td>
<td>CEP290</td>
<td>Leber’s congenital amaurosis</td>
<td>2/3</td>
<td></td>
</tr>
<tr>
<td>Casimersen</td>
<td>PMO⁵</td>
<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
<td>3</td>
<td>Submitted for registration</td>
</tr>
<tr>
<td>SRP-4043</td>
<td>PMO⁶</td>
<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Tominersen</td>
<td>ASO</td>
<td>HTT</td>
<td>Huntington's disease</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Aganirsen</td>
<td>ASO</td>
<td>IRS1</td>
<td>Ischemic central retinal vein occlusion, neovascular glaucoma</td>
<td>2/3</td>
<td></td>
</tr>
<tr>
<td>Nedosiran</td>
<td>siRNA</td>
<td>LDHA</td>
<td>Primary hyperoxaluria</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Pelacarsen</td>
<td>ASO</td>
<td>LPA</td>
<td>Cardiovascular disease and lipoprotein(a)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Inclisiran</td>
<td>siRNA</td>
<td>PCSK9</td>
<td>Atherosclerotic cardiovascular disease</td>
<td>3</td>
<td>Submitted for registration; approved by EMA</td>
</tr>
<tr>
<td>Fitisiran</td>
<td>siRNA</td>
<td>SERPINC1</td>
<td>Hemophilia A/B</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Tofersen</td>
<td>ASO</td>
<td>SOD1</td>
<td>Amyotrophic lateral sclerosis</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Patisiran</td>
<td>siRNA</td>
<td>TTR</td>
<td>hATTR cardiomyopathy, polyneuropathy</td>
<td>3</td>
<td>Approved for hATTR polyneuropathy</td>
</tr>
<tr>
<td>Vutrisiran</td>
<td>siRNA</td>
<td>TTR</td>
<td>hATTR cardiomyopathy, polyneuropathy</td>
<td>3</td>
<td>Submitted for registration for hATTR polyneuropathy</td>
</tr>
<tr>
<td>AKCEA-x TTR-LF</td>
<td>ASO</td>
<td>TTR</td>
<td>hATTR cardiomyopathy, polyneuropathy</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Volanesorsen</td>
<td>ASO</td>
<td>APOC3</td>
<td>Familial chylomicronemia syndrome, familial partial lipodystrophy</td>
<td>3</td>
<td>Completed; approved by EMA, rejected by FDA</td>
</tr>
<tr>
<td>Oblimersen</td>
<td>ASO</td>
<td>BCL2</td>
<td>Malignant melanoma, DLBCL</td>
<td>2/3</td>
<td>Terminated</td>
</tr>
<tr>
<td>Custirsen</td>
<td>ASO</td>
<td>CLU</td>
<td>Castrate-resistant prostate cancer</td>
<td>3</td>
<td>Terminated; failed primary endpoint¹</td>
</tr>
<tr>
<td>Suvodrinsen</td>
<td>ASO⁵</td>
<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
<td>2/3</td>
<td>Terminated; lack of efficacy</td>
</tr>
<tr>
<td>Drisapersen</td>
<td>ASO⁵</td>
<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
<td>3</td>
<td>Terminated; rejected by FDA due to safety concerns and lack of efficacy</td>
</tr>
<tr>
<td>Alicaforsen</td>
<td>ASO</td>
<td>ICAM-1</td>
<td>Crohn’s disease, pouchitis</td>
<td>3</td>
<td>Completed; failed primary endpoint but development ongoing on enema formulation for UC</td>
</tr>
<tr>
<td>Pegpleranib</td>
<td>Aptamer</td>
<td>PDGFB</td>
<td>Wet age-related macular degeneration</td>
<td>3</td>
<td>Terminated; failed primary endpoint</td>
</tr>
<tr>
<td>Aprinocarsen</td>
<td>ASO</td>
<td>PRKCA</td>
<td>Non-small cell lung cancer</td>
<td>3</td>
<td>Completed; development discontinued due to lack of efficacy¹</td>
</tr>
<tr>
<td>Mongersen</td>
<td>ASO</td>
<td>SMAD7</td>
<td>Crohn’s disease</td>
<td>3</td>
<td>Terminated; lack of efficacy⁹</td>
</tr>
<tr>
<td>Trabedersen</td>
<td>ASO</td>
<td>TGFB2</td>
<td>Anaplastic astrocytoma, glioblastoma</td>
<td>3</td>
<td>Terminated</td>
</tr>
<tr>
<td>Revusiran</td>
<td>siRNA</td>
<td>TTR</td>
<td>hATTR cardiomyopathy</td>
<td>3</td>
<td>Completed; development discontinued due to imbalance of mortality in treatment arm³</td>
</tr>
</tbody>
</table>

ᵃ Gene nomenclature per HGNC guidelines;ᵇ Sepofarsen binds the preRNA of the CEP290 gene (with the p.Cys998X mutation) and restores expression of wild-type CEP290;ᶜ Casimersen binds DMD pre-mRNA at exon 45 to promote exon 45 skipping in DMD gene;ᵈ SRP-4043 binds DMD pre-mRNA at exon 53 to promote exon 53 skipping in DMD gene;ᵉ Suvodrin and drisapersen binds DMD pre-mRNA at exon 51 to promote exon 51 skipping in DMD gene;¹ Chi et al., 2017;⁷ Sands et al., 2020;⁷ Sutherland et al., 2020;⁷ Paz-Ares et al., 2006. ASO, antisense oligonucleotide; DLBCL, diffuse large B-cell lymphoma; hATTR, hereditary transthyretin-mediated amyloidosis; PMO, phosphorodiamidate morpholino oligomer; siRNA, small interfering RNA; SSO, splice switching oligonucleotide; UC, ulcerative colitis
partially complementary sites in mRNAs, resulting in translational repression and mRNA destabilization (Felekissi et al., 2010).

In contrast to the ONT classes described above (ASOs, siRNA, miRNA-mimics) that target RNA via base pairing, aptamers and immunostimulatory CpG oligos target soluble proteins or receptors and exert biological activity by adopting specific three-dimensional (3D) conformations (Hanagata, 2017; Keeffe et al., 2010; Zhu and Chen, 2018). Other nucleotide-based therapeutic approaches such as mRNA therapy and gene editing applications that employ guide RNA (e.g., CRISPR/Cas9) are not commonly classified as ONTs, with size being one distinguishing factor (Fig. 1). Guide RNAs for CRISPR-mediated gene editing are typically 100 nucleotides in length (Wang et al., 2019) and require complexing with Cas9 to exert pharmacology. Therapeutic mRNAs can contain a nucleotide cap, 5' and 3' untranslated regions, an open-reading frame, and a 3' poly(A) tail, totaling more than 1000 nucleotides (Jackson et al., 2020). In 2020/21, two mRNA-based therapies, Moderna’s mRNA-1273 and Pfizer/BioNTech’s BNT162b2, attracted worldwide interest as vaccines authorized for emergency use for the prevention of COVID-19. Other mRNA-based vaccines have been tested in early clinical trials including those targeted against rabies, influenza, cytomegalovirus, and zika (Alberer et al., 2017; Feldman et al., 2019; Pardi et al., 2018). Although these examples are out of scope for this review, they highlight the rapidity with which therapeutics containing oligonucleotides can be developed against a particular target and the breadth of possible therapeutic uses.

Despite several regulatory approvals, formal regulatory guidance for ONT development is not available (Hirabayashi et al., 2021). Oligonucleotides are generally chemically synthesized and are classified as new chemical entities (NCE). The nonclinical development of marketed ONTs has generally adhered to the regulatory guidance in the International Conference on Harmonization (ICH) M3(R2) “Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals” (FDA, 2010). However, certain biochemical properties of ONTs warrant additional consideration by sponsors, including potential off-target sequence effects, unique metabolic pathways, and evaluation of immunogenicity as described in ICH S6(R1) “Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals” (EMA, 2011). A cross-company survey conducted in 2018 reported that most ONT developers regard many regulatory documents used for small molecules to be less relevant for ONTs (Tessier et al., 2021). In the absence of formal guidelines, support and examples of best practice can be found in a series of white papers published by the Oligonucleotide Safety Working Group (OSWG) (Alton et al., 2012; Berman et al., 2014, 2016; Capaldi et al., 2017; Cavagnaro et al., 2014; Marlowe et al., 2017; Schubert et al., 2012).

1.2 Microphysiological systems (MPS) in ONT development

Since ONTs are generally specific for human gene sequences, limited homology in nonclinical species often impacts PD response or results in lack of pharmacological activity, necessitating either transgenic animals or parallel development of a surrogate species-specific ONT to generate nonclinical pharmacology and toxicology data. An alternative strategy is to implement human in vitro models to characterize clinical candidates. Standard in vitro models typically employed during small molecule drug development have proven difficult to translate to ONT applications, motivating the need for advanced models with greater complexity, such as MPS.

The International Consortium for Innovation and Quality in Pharmaceutical Development Microphysiological Systems Affiliate (IQ MPS) defines MPS as an extension of a traditional two-dimensional (2D) in vitro culture system that includes several of the following features: “a multi-cellular environment within biopolymer or tissue-derived matrix, a 3D structure, mechanical factors such as stretch or perfusion (e.g., breathing, gut peristalsis, flow), primary or stem cell-derived cells, and/or inclusion of immune system components” (Fabre et al., 2020). Traditional in vitro culture models generally contain cells relevant to an organ of interest. MPS developers have made significant progress in the development of organotypic culture models, here defined as tissue or organ analogs often comprised of isolated tissue fragments, terminally differentiated primary cells or cell lines, which are aimed to better mimic the physiology of a single organ (i.e., liver, kidney, lung etc.). An example of extending beyond the 2D in vitro culture system involves utilizing primary human cells in a microfluidic device that can recapitulate physiological properties (e.g., shear-stress) not present in traditional in vitro systems. This type of microfluidic device, often referred to as an “organ-on-a-chip”, has recently been utilized in the oligonucleotide space (Lidberg et al., 2021; discussed in more detail in Section 4.5 below). In the case of Lidberg et al. (2021), animal models would not have added value, as the human transcriptome was requisite to query the interplay between pharmacology, off-target binding, and toxicology (Lidberg et al., 2021). The defining characteristics of an MPS for the purposes of this review are depicted in Figure 2A; tissues currently being evaluated in the ONT space are highlighted (Fig. 2B), and pharmacological, toxicological, and absorption, distribution, metabolism, and excretion (ADME) endpoints are considered (Tab. 3). This review does not intend to provide details on available organotypic models, as these are covered elsewhere. The reader is directed to the following references for information on MPS models of skin (Hardwick et al., 2020), spleen (Miller et al., 2020), kidney (Phillips et al., 2020), liver (Baudy et al., 2020; Ribeiro et al., 2019), eye (Bai and Wang, 2020; Wright et al., 2020), central nervous system (CNS) (Haring et al., 2017; Lee and Leong, 2020), lung (Ainslie et al., 2019), bone marrow (Santos Rosalem et al., 2020), gastrointestinal system (Peters et al., 2020), tumor microenvironment (Trujillo-de Santiago et al., 2019), and skeletal muscle (Ananthakumar et al., 2020; Truskey, 2018).

This review is separated into particular contexts of use for ONTs (i.e., ADME, pharmacology, toxicology), recognizing that different requirements arise depending on the questions being asked. Aspects of MPS that are well or poorly suited to address the unique challenges of ONT development are summarized. Discussion includes potential areas where MPS might be leveraged during ONT discovery and development. As will be discussed in detail, MPS may offer an opportunity to optimize ADME properties of ONTs (delivery, exposure, or metabolic sta-
Drug metabolism and pharmacokinetics

During drug discovery and development, it is required to characterize what the body does to the drug, given that exposure drives biological effect. A comprehensive understanding of drug metabolism and pharmacokinetics (PK) can define plasma and tissue exposures producing therapeutic or toxic effects and ultimately predict human efficacious dose levels. Human efficacious dose levels can be predicted upon integration of pharmacology data together with in vitro, in silico, and PK data from animal models characterizing the clearance, distribution, and excretion of new therapeutics. By generating relationships between PK and PD, it is possible to predict an efficacious human dose and characterize a margin of safety to toxic doses identified in nonclinical toxicology studies. Typically, in small molecule drug development, drug levels in the plasma are used to estimate human dose levels and regimens. Unlike small molecule drugs, there is a considerable delay between maximal ONT levels in the plasma and maximal observed PD, which is driven by tissue distribution. Tissue PK is generally not measurable in humans and therefore cannot be used to build confidence in the utility of animal models or in vitro systems for predicting PK/PD relationships in humans. In general, drug metabolism and pharmacokinetics properties of ONTs...
are sequence independent and are often predictable within platforms that employ consistent chemical modifications and design, but there are still opportunities to apply MPS to address mechanistic ADME questions as outlined in Table 3. Specific questions related to ADME including species concordance are not unique to ONTs. However, the unique properties of ONTs, including mechanisms of uptake, longevity of tissue PK, intracellular distribution, and excretion are highly dependent on specificity and efficiency of uptake and offer an opportunity to apply MPS where standard in vitro models have failed to be predictive.

### 2.1 Absorption and distribution

The absorption and therefore oral bioavailability of ONTs is low. To enter systemic circulation, ONTs are often administered either by subcutaneous (s.c.) injection or intravenous (i.v.) infusion (Fig. 1). Local administration is a common approach to achieve sufficient exposure in certain tissues inaccessible to systemic administration of ONTs. For example, intrathecal (i.t.) injections are used to enable delivery and distribution to CNS, and intravitreal (i.v.t.) administration bypasses the need to cross the blood-retina barrier and maximizes distribution to the eye (Tab. 1).

Absorption, biodistribution, and plasma protein binding properties are generally governed by ONT class, chemical modifications, and size. The distribution properties of FDA-approved ONTs to-date have been evaluated in nonclinical studies using radiolabeled (^{14}C or ^{3}H) ONTs. After systemic administration, the highest concentrations of PS-modified ASOs (PS-ASOs) can be found in the kidneys and liver, followed by spleen and bone marrow (Geary et al., 2015), whereas very low to negligible exposure is observed in other compartments, such as the CNS and testes. Uptake differs between cell types within a tissue; in the kidney, the highest concentration of PS-ASOs is found in the cortical proximal tubular cells, whereas in the liver, Kupffer and liver sinusoidal endothelial cells show the highest exposure (Debacker et al., 2020; Graham et al., 1998). The extent of kidney accumulation is dependent on the size and degree of chemical modification of ASOs. Chemical modifications of ASOs modulate their plasma protein binding and absorption properties (Andersson and den Besten, 2019). In general, PS-ASOs are highly protein-bound, avoiding renal filtration. Following subcutaneous delivery, tissue absorption of PS-ASOs is generally higher than that of uncharged ASOs (PNAs and morpholinos), which are rapidly cleared from plasma (Geary et al., 2015). Size and charge of ASOs often prevent their transit across the blood-brain barrier (BBB) and subsequent absorption by the CNS, which may be beneficial but also pose a delivery challenge that can potentially

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<td>Influence of disease state on ADME or toxicity susceptibility?</td>
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Tab. 3: Potential contexts of use where MPS may address nonclinical questions/concerns

Note: MPS context of use Nonclinical questions or concern

Liver MPS (and multi-organ chips) Productive uptake of liver-targeting ONTs
Target engagement/PD and efficacy endpoints of liver-targeting ONTs
Mechanisms of biliary excretion
Relationship between liver PD and extrahepatic disease modification of liver-targeting ONTs
Mechanisms of toxicity and translation to human

Kidney MPS Impact of disease state on clearance
Mechanisms of toxicity and translation to human

Vascular/lymphatic MPS (Osaki et al., 2018) Mechanism of systemic distribution
Contribution of endothelium to ONT-induced immunomodulation and relevance to human

BBB MPS Retention of i.t. administered ONTs
Mechanisms of transport across BBB into CNS for s.c. or i.v. administered CNS-targeted ONTs
Exposure response relationship for PD markers
Mechanisms of toxicity and translation to human

Nervous system MPS PD/efficacy for ONTs indicated for neurodegenerative disease
Biomarker evaluation in ex vivo CSF

Skin MPS ONT retention in the injection site?
Metabolism at the injection site?
Skin absorption for topical ONTs
Mechanism of injection site reactions and species relevance

GI/oral mucosa MPS Gut/oral absorption of ONTs
Target engagement/PD and efficacy endpoints

Ocular MPS Mechanisms of ONT retention upon i.v.t. administration
Target engagement/PD and efficacy endpoints

Skeletal muscle MPS PD/efficacy for DMD exon skipping ONTs

All Metabolic stability in tissues?
Metabolic competency and metabolite profiling?
Pharmacologically active or toxic metabolites?
Species differences in ADME, pharmacology, toxicity?
Influence of disease state on ADME or toxicity susceptibility?
be overcome by evaluating different formulations, modifications, and conjugates of ONTs using MPS of the neurovascular unit as discussed in later sections.

In contrast to the uptake of PS-ASOs into several different cell types, siRNA requires special formulation, i.e., lipid nanoparticles (LNP), or receptor ligand conjugation for delivery to tissues after systemic administration. Without these, siRNA is rapidly excreted via the urine (Lin and Tam, 2015). The carbohydrate N-acetylgalactosamine (GalNAc) is an endogenous ligand of the asialoglycoprotein receptor (ASGPR), a rapidly recycling cell surface receptor that is abundantly expressed on hepatocytes of most mammalian species. GalNAc-conjugated siRNA and PS-ASOs show impressively enhanced cellular uptake that translates to enhanced activity in hepatocytes and enhanced potency in vivo (Debacker et al., 2020). For 2'-methoxyethyl (MOE) gapmers, GalNAc conjugation can improve ONT potency for hepatocyte targets in humans by 20- to 30-fold, although in vitro systems fail to recapitulate the observed magnitude by a log order (Viney et al., 2021). In vitro models with meaningful physiological relevance offered by MPS may enable evaluation, exploration, and optimization of the processes involved in uptake and intracellular release of ONTs.

The targets of ONTs reside within the nucleus or cytoplasm of the cell, therefore biological activity is dependent on internalization to the cell(s) of interest. Intracellular target localization can also mean that additional delivery is required. ONT uptake and trafficking is dependent on multiple factors related to the ONT (concentration, sequence, modality, chemical modifications, dosing route) and the target (tissue or cell type). Knowledge of the mechanism of ONT uptake into cells, including extent, subcellular distribution, and whether free or bound is relevant for establishing exposure response relationships. Whereas small and non-charged molecules can enter cells through passive diffusion or specific transporters, large charged or polar molecules like ONTs typically enter cells through endocytosis (Yang and Himner, 2015). Endocytosis is energy-dependent and involves internalization of cell surface proteins and extracellular contents through plasma membrane invagination. Resulting membrane-bound endocytic vesicles of varying size are then trafficked through the endo-lysosomal system where contents are recycled to the cell surface, degraded in lysosomes, or released into the cytosol. The pathways for endocytosis are cell-type dependent and include macropinocytosis, caveolar endocytosis, and clathrin-dependent or independent endocytosis. Characterizing these processes within an MPS model would be beneficial since understanding the mechanisms of ONT internalization and trafficking within cells can aid in improving design, delivery, and efficiency of endosomal escape (Croke et al., 2017b; Juliano, 2016).

In vitro studies often fail to reproduce in vivo ONT uptake. Many different cells take up ASOs in vitro but do not result in the level of activity observed in vivo (Koller et al., 2011). Recaptulating the in vivo situation is even more important when evaluating and optimizing different conjugation approaches or delivery systems. With improved local context and the appropriate physiological mechanics preserved, MPS for tissues and cell types of interest should serve as the ideal bridge between higher throughput 2D systems and the intact organism, allowing critical cross-species translation and improved understanding.

### 2.2 Metabolism

Both ASO and siRNA undergo metabolism through exo- and endonucleases ubiquitously distributed in plasma and tissues rather than through standard drug metabolizing enzymes. Exonucleases cleave the ends of nucleotide strands, resulting in the release of mononucleotides and formation of truncated metabolites, while endonucleases cleave internally resulting in nucleotide strands of varying lengths. Chemical modifications reduce metabolic degradation of ASOs and siRNA. For example, current chemistries of GalNAc-siRNA demonstrate high stability and, thus, limited potential for major circulating metabolites (Nair et al., 2017). The GalNAc-siRNA design used in approved ONTs includes an overhang on the antisense strand, which contains two PS linkages to confer resistance to exonucleases (de Fougerolles et al., 2007). Loss of a single nucleotide from the 3' end of the antisense strand (AS), annotated as 3'N-1 (AS), is an often-observed metabolite and may be pharmacologically active. Metabolite identification studies on givosiran, approved in 2020 for acute hepatic porphyria, revealed that 3’N-1 givosiran was a major circulating metabolite formed to varying extents in rat, monkey and human (Agarwal et al., 2020). Other RNAi therapeutics have demonstrated that the predominant drug-related material in the circulation is full-length parent drug with limited contribution of circulating metabolites. Of note, the FDA clinical pharmacology reviews for approved ASOs often label metabolites as nuclease-derived and not pharmacologically active, suggesting active metabolites should be addressed on a case-by-case basis.

Species concordance in metabolite formation or whether there are any disproportionate or pharmacologically active human metabolites should be considered. Since the majority of metabolism occurs in the target or eliminating tissues, it is nearly impossible to obtain this information clinically. The current industry standard relies on a combination of in vitro and in vivo models to evaluate metabolite profiles in plasma and tissues. In the absence of relevant human data, confidence may be built through in vitro-to-in vivo extrapolation with nonclinical models. In the case of metabolically stable ONTs, physiologically relevant MPS that retain high nuclease activity for a prolonged period (weeks to months) may be useful for understanding metabolite formation across tissues and species. It is also important to understand the metabolism of any linkers or conjugates. As an example, GalNAc-ASOs undergo asialoglycoprotein receptor (ASGR)-mediated endocytosis and subsequent rapid GalNAc cleavage by β-N-acetylgalactosaminidase (Shemesh et al., 2016).

### 2.3 Excretion

Clinically, understanding the excretion pathways for ONTs relies on measurement of intact parent in urine since limited intact parent is observed in feces. Biliary excretion of intact ASGR ligands such as GalNAc has been reported (0.5-15%) (Ryvniak, 1987; Thomas and Summers, 1978). It is possible that ONTs that are re-
The unique pharmacological properties of ONTs include 1) “event-driven” (i.e., decreasing the cellular protein levels to reduce protein function) vs “occupancy-driven” pharmacology (i.e., applying high concentrations of inhibitor to reduce protein function), 2) often poor cross-reactivity between species, 3) restricted number of cell types in which full activity can be achieved, 4) delayed onset to maximum pharmacology, and 5) long effect duration. While screening approaches to assess pharmacology in nonclinical species. For such cases, surrogate molecules of the same chemistry and design showing sufficient on-target activity and comparable safety in the animal model of choice can be employed during nonclinical development. Four FDA-approved ASOs including mipomersen and all three FDA-approved ONTs for Duchenne muscular dystrophy (DMD) (eteplirsen, golodirsen, and viltolarsen) (Tab. 1) included data from surrogates in their pharmacology packages. The pharmacology of ONTs for DMD has primarily been investigated using non-human primates, human primary cells, or the mouse mdx model, which harbors a nonsense mutation in mouse Dmd exon 23 producing dystrophin deficiency. Exon skipping via splice-switching oligonucleotides (SSOs), specifically at exons 51 and 53 of human DMD pre-mRNA, addresses the genetic basis of the human disease but is irrelevant to exon skipping in mice (Echigoya et al., 2017). Species specificity in the genetics of DMD motivated the use of surrogate ONTs targeting different exons in the mouse Dmd gene for pharmacology assessment. However, the parallel nonclinical development of a surrogate ONT requires significant cost and time resources. A suitable MPS for muscle tissue incorporating healthy or diseased patient cells could serve as a pharmacological model for these types of drug candidates and limit the use of animal disease or efficacy models.

To guide human dose estimation, transgenic mice expressing the human gene can be used to assess in vivo potency, taking the tissue and cell type-specific uptake into account. However, due to potential differences in cellular uptake and processing (e.g., endosomal release and metabolism) of ONTs between rodents and humans, cell-based models using patient-derived cells, particularly cells from patients harboring the disease of interest, are attractive for pharmacological assessment of ONTs. In the absence of patient primary cells, human induced pluripotent stem cells (hiPSCs) may serve as a suitable alternative. Patient-derived hiPSCs harboring specific genetic mutations can be used to establish clinical proof-of-concept and aid in identifying responsive patient subpopulations during nonclinical development.

3.2 Pharmacological tissues of interest

This section highlights several examples of diseases currently indicated for FDA-approved ONTs (Tab. 1) or under investigation (Tab. 2). A comprehensive review of ONT pharmacology and associated targets currently being explored in early clinical trials has been published (Crooke et al., 2020a).

3.2.1 Liver

As a principal therapeutic target organ for ONTs, the liver represents an attractive focus for in vitro modeling to gain mechanistic insights and model disease. The liver is a tissue of interest for the MPS community (Baudy et al., 2020), particularly for drug pharmacology testing (Yang et al., 2020). Multiple liver-related diseases have been successfully treated with ONTs including hereditary transthyretin amyloidosis (hATTR), acute hepatic...
porphyria (AHP), and primary hyperoxaluria type 1 (PH1). MPS may enable development of transformational therapies for liver fibrosis and steatosis, where traditional model systems (in vitro and in vivo) fail to recapitulate the severity or complexity of these multi-faceted diseases. While the majority of ONTs presently target hepatocytes, MPS would ideally contain both parenchymal and non-parenchymal cells to better mimic the in vivo environment. Hepatocytes can be targeted by ONTs in several ways, including through LNP encapsulation or ligand conjugation (e.g., GalNac) (Fig. 1). Many approved ONTs target the liver (Tab. 1), owing mainly to its amenability to selective targeting and ability to achieve PD with or without conjugation, particularly for ASOs. The pharmacology of liver-directed ONTs has primarily been established using animal models (Tab. 1). However, animal models often fail to reproduce hallmarks of the disease, limiting endpoints to PD readouts of decreased target expression by qPCR. For example, rodent models harboring human transthyretin (TTR) mutations failed to reproduce the amyloid build-up characteristic of the pathology of hATTR (Sousa et al., 2002). It is possible that human MPS can address pitfalls of current animal models of disease and be used to generate clinically relevant functional endpoints.

In vitro models of the human, rodent, monkey, and dog liver are commercially available, presenting the opportunity to compare findings across species. Liver models range in complexity from simple 2D hepatocyte cultures to more advanced 2D or 3D co-culture systems employing other liver-resident cells like Kupffer and endothelial cells. The potential complexities of MPS are illustrated in Figure 2A. Hepatocyte spheroids more faithfully recapitulate the proteome of human liver tissue relative to 2D culture (Bell et al., 2016), a property that is attractive for clinical translation of pharmacology assessment. Physiologically-relevant shear flow and low fluid volumes in combination can also sustain the phenotype and function of primary human hepatocytes beyond that of static cultures (Haque et al., 2016). Primary human hepatocytes have proven to be a workhorse for preclinical drug development. Their limited proliferation is advantageous from an ongoing research into ONTs that promote DMD exons 51-53. Demonstrations of pharmacology are incompatible with exon skipping for treating human DMD as discussed. Transgenic mouse models of SMA have proven useful for preclinical ONT development, but it remains a challenge that SMA is a human-specific genetic disease with varied disease presentation dependent on the expression of full-length SMN2, which is not encoded in the mouse genome (Monani et al., 2000). The lack of appropriate animal models that recapitulate the genotype and phenotype of human genetic disease motivates an alternative approach.

MPS may accurately model relevant disease pathology using human cells harboring the genotype of interest. It is likely that ongoing research into ONTs that promote DMD exon skipping will continue to rely on in vitro demonstrations of pharmacology. Rudimentary 2D cultures of human primary or immortalized hepatocytes can be used to characterize target expression by qPCR, but these models lack phenotypic relevance to study functional consequences of PD and study the degree of DMD modulation required for meaningful biological effect. As an alternative, human skeletal muscle tissue can be generated in vitro through 3D culture of primary human myogenic cells or myoblasts or from stem cell-derived myoblasts (Gholobova et al., 2018; Urciuolo et al., 2020). Functional endpoints like contractility (Nagashima et al., 2020) and contraction and displacement of in vitro human skeletal muscle (Takahashi et al., 2018) may enable correlation of PD to disease modification in vivo. Modeling of SMA in vitro has been limited to rudimentary 2D cultures of stem cell-derived motor neurons to date (Ohuchi et al., 2016; Zhang et al., 2017).
Emerging approaches using complex in vitro co-culture models incorporating other cell types involved in SMA including astrocytes (Adami and Bottai, 2019) or organoids capable of differentiating to multiple spinal cord cell lineages including motor neurons (Hor et al., 2018) may enable modeling the multi-cellular degenerative aspects of SMA. However, the ability of musculoskeletal MPS to achieve productive uptake of ONTs is yet to be demonstrated. Coincidentally, because the differentiation process can take up to weeks of culture, this makes stem cell-derived musculoskeletal MPS amenable to treatment with ONTs to take advantage of their long duration of pharmacology. Further, the addition of ONT at specific periods of musculoskeletal development may be investigated to identify at what point of disease progression the drug is most likely to provide clinical benefit.

3.2.3 Central nervous system
Nervous system disorders with a genetic basis are challenging to model in animals or in vitro, the latter due to the impossibility of sourcing primary cells from patients. Stem cell technology promises to address the challenge of developing CNS disease models. Several reports on the derivation of hiPSCs from Huntington’s disease (Csobonyeieva et al., 2020) and amyotrophic lateral sclerosis patients (Zhao et al., 2020) add to the available toolset for modeling these genetic neurodegenerative diseases in vitro. Neurodevelopmental disease modeling has been a focus of ongoing brain organoid research for the past decade (Wray, 2020) and may soon enable assessment of ONT pharmacology. Advances in MPS now allow measurement of cerebrospinal fluid (CSF) production from choroid plexus organoids (Pellegrini et al., 2020) and real-time sampling of blood and neural compartments from a neurovascular unit on a chip (Brown et al., 2016). Both technologies combined may aid real-time sampling of engineered CSF fluid to measure pharmacological response and exposure to ONTs in a manner that is analogous to CSF biomarker assessment in preclinical and clinical studies.

3.2.4 Ocular
The first two FDA-approved ONTs, fomivirsen and pegaptanib, are indicated for ocular diseases. Since their approval, numerous advances have been made in ocular MPS models and several new ocular indications have been explored in the clinic (Tab. 2). Patient-derived or manipulated hiPSCs can be cultured in 3D embryoid bodies that can be subsequently differentiated to retinal organoids mimicking aspects of the retina (Capowski et al., 2019). Retinal organoids are amenable to disease modeling and can be readily generated from Leber’s congenital amaurosis patient cells (Li et al., 2019). Other components of the eye including the retinal vasculature, lens, and cornea can be modeled in vitro (Bai and Wang, 2020) enabling characterization of ONT pharmacology for aspects of ocular disease.

3.2.5 Gastrointestinal tract and skin
Many diseases of the gastrointestinal tract and skin are characterized by chronic inflammation that is difficult to mimic in animal models or in vitro. Better preclinical models for inflammatory diseases like inflammatory bowel disease (IBD) and psoriasis may be warranted to effectively validate new drug targets and evaluate ONTs preclinically. In vitro models of IBD necessitate incorporating multiple cell types involved in normal gut function (epithelium, endothelium) including immune cells essential to disease progression. In vitro gut models have been extensively reviewed elsewhere (Peters et al., 2020) and may prove useful to evaluate the feasibility of achieving pharmacology with ONTs directed to the GI tract. MPS of the gut or skin could also evaluate the feasibility of oral or topical ONT delivery for psoriasis. Data generated from engineered human gut mucosa or skin models can be paired with data from nonclinical species showing drug distribution upon oral or topical delivery. Given the lack of efficacy of oral and topical ONTs in the clinic (Tab. 2), it is crucial to understand the feasibility of achieving a pharmacological effect via these delivery routes using a combination of in vivo and in vitro tools.

3.2.6 Cancer
Several ONTs have been investigated for cancer indications in clinical trials (Tab. 2). Targeted oncology therapies have shown promise for enhancing survivability beyond that of traditional chemotherapy though progress is still needed. The response duration and long half-life of ONTs can address this shortcoming of small molecules, but clinical trial results have largely shown no therapeutic benefit over the standard of care for investigational ONTs. Cancer remains a challenging indication owing to the extremely varied disease presentation, genetic heterogeneity, and tissue-specific modes of tumor promotion and metastasis. Complex diseases necessitate complex multicellular modeling approaches, and the same is likely true for anti-cancer ONTs. Multicellular cancer MPS may be able to drive emergent phenotypes and uncover novel oncology targets inaccessible to traditional 2D approaches. MPS has received little attention as a tool for assessing anti-tumor potential of ONTs, but an MPS model employing 3D culture of tumor spheroids and vascular endothelium demonstrated PD and in vitro inhibition of angiogenesis with siRNA targeting VEGFR (Lee et al., 2021). Anti-tumor proof-of-principle with ONTs using clinically validated targets is an important first step toward achieving clinical benefit of ONTs.

4 Safety
This section highlights several ONT toxicities, which typically manifest as injection site reactions, immunomodulation or thrombocytopenia driven by plasma exposures, or liver or kidney toxicity driven by drug accumulation. MPS carry specific advantages critical to ONT safety assessment. First, MPS enables long-term cell culture essential to study potential PD or toxicologic effects resulting from longer exposure duration. Second, MPS recreates tissue microphysiology mimicking in vivo-like transcript expression profiles, enabling assessment of PD and hybridization-dependent toxicity and consequences. Finally, MPS enables recapitulation of complex multi-cellular toxicological effects like inflammation to evaluate mechanisms of nonclinical find-
ings and relate them to human risk. The potential utility of MPS to address nonclinical toxicology questions during ONT development are outlined in Table 3.

4.1 On- and off-target toxicities
Safety liabilities of ONTs can be classified into Watson-Crick hybridization-dependent or hybridization-independent effects. Hybridization-dependent toxicities can arise from on-target and off-target pharmacology (Kornbrust et al., 2013; Lindow et al., 2012); the latter from the ONT binding unintended RNA targets. The frequent lack of cross-reactivity of human therapeutic candidates in toxicological species complicates hazard identification and quantitative risk assessment of potential on-target and off-target safety concerns in animals. Animal active surrogate ONTs can be utilized, but interpretation of such studies could be hampered by a different overall safety profile of the surrogate molecule and the target having different biological roles between species. Human MPS that recapitulate in vivo expression of on- and off-target transcripts of interest and their biological function would be of great value for assessing hybridization-dependent ONT toxicities.

4.2 Complement activation
For PS-ASOs, non-hybridization toxicities can be dependent or independent of sequence. An example of the latter is prolongation of activated partial thromboplastin time, which results from the PS backbone interacting with the Tenase complex in the coagulation cascade (Sheehan and Phan, 2001). Complement factor H is an endogenous activation inhibitor that can be bound by ASOs in a PS backbone-dependent manner, lowering the threshold of activation and inducing alternative complement activation. These effects are driven by the maximum plasma concentration (C_{max}) and are transient since plasma clearance is rapid. With increased potency and C_{max}-lowering dosing regimens and administration routes, such critical C_{max} thresholds are now rarely exceeded in clinical studies. Cynomolgus monkeys are particularly sensitive to complement activation, which can progress to vascular inflammation in chronic studies (Henry et al., 2016). While MPS systems aimed at modeling immune system responses are emerging (Miller et al., 2020), the monitoring of complement activation in MPS will require the addition of complement proteins, potentially through incorporating effluent from hepatocytes (where complement proteins are synthesized). This endeavor would benefit from connection of multiple chips to model a complex immune response but requires significant effort and optimization and is therefore a lofty goal.

4.3 Inflammation
In contrast to plasma exposure-driven toxicities, many ONT-induced toxicities are sequence-dependent. This includes cytotoxicity and proinflammatory effects observed in high-exposure organs such as administration site, liver, and kidney. An example of the latter is PS-ASO-induced glomerulonephritis. Mice are particularly susceptible to PS-ASO-induced glomerulonephritis that is caused by C_{max}-driven immunostimulation mediated by CpG-mediated toll-like receptor activation (Frazier and Obert, 2018). Despite avoiding CpG motifs in the design phase, some ONTs induce proinflammatory effects that manifest in several different ways, including injection site reactions (ISRs), infusion reactions, and sometimes thrombocytopenia (TCP). These effects are dose-dependent and can occur at different time points after administration. Unfortunately, clinical proinflammatory risk is often difficult to assess using animal models. Here, advanced and multifaceted MPS would have great potential.

ONTs with minimal chemical modifications require delivery in a protective formulation like LNPs, which are often associated with proinflammatory effects. The first approved siRNA, patisiran (Tab. 1), requires premedication with paracetamol, corticosteroids, and antihistamines prior to administration. However, advances in stabilizing chemistry and conjugation ensure that most siRNA candidates no longer require delivery vehicles or immunomodulator pretreatment.

Thrombocytopenia (TCP) is characterized by low concentrations of circulating platelets and has occasionally been observed in non-human primate (NHP) toxicity studies with ASOs. ASO-induced TCP in cynomolgus monkeys can sometimes be severe (Henry et al., 2017). Historically, severe TCP had only been observed in a few NHP toxicology studies for oncology programs. However, in 2016, severe TCP was reported in Phase 3 studies for ASO gapmers volanesorsen and inotersen (Crooke et al., 2017a) and for the SSO drisapersen\(^1\). These TCP events only occurred in the highest dose group, and platelet counts recovered after drug cessation. Experiments with non-CpG ASOs with therapeutically common modifications indicate no effect on the bone marrow and thrombopoiesis and have largely ruled out ONT-induced activation of platelet factor-4 (PF4) as a mechanism (Sewing et al., 2017; Narayanan et al., 2018). Instead, TCP has been attributed to a combination of pro-inflammatory effects at high drug levels and to patient susceptibility factors. This has been supported by the high frequency of severe TCP after administration of ASOs in cynomolgus monkeys from a Mauritian inbred population compared to a non-Mauritian population, indicating a genetic component (Andersson and den Besten, 2019).

The involvement of the endothelium in PS-ASO-related vasculitis and glomerulonephritis requires incorporating endothelial cell-lined tubules and shear flow to enable characterization of drug-induced effects on endothelial complement to address safety liabilities related to ASO-induced immunostimulation (Sfriso et al., 2018). Co-culture of endothelial cells with human peripheral blood mononuclear cells (Lubbers et al., 2017) would enable characterization of pro-inflammatory effects or immunostimulation by ASOs, as previously demonstrated with co-culture of PBMCs with intestinal epithelial cells to model gut inflammation (Miller et al., 2020). While these complex in vitro models do not meet our definition of MPS, they do provide a rationale for including an immunocompetent component in MPS for ONTs. Inflammation involves crosstalk between multiple cell types, thus

a model consisting of multiple cell types involved in complement activation and vasculitis would be necessary to parse mechanisms of and evaluate species-specific susceptibility to ONT-associated immunomodulation.

An in vitro model of platelet production and activation in a microfluidic platform would be useful for interrogating mechanisms of and species differences in susceptibility to ONT-mediated TCP. Although current research rules out mechanisms of ONT-induced TCP involving decreased platelet production from megakaryocytes or off-target platelet activation, models of these processes may still prove useful for future ONT development (Visentin and Liu, 2007). Platelets can be readily produced from megakaryocytes in MPS (Blin et al., 2016), and such models can be used to determine whether the mechanism of ONT-mediated TCP is due to decreased or dysfunctional platelet production. A thrombosis-on-a-chip model (Barrile et al., 2018) could also be used to characterize any off-target ONT-induced effects on direct platelet activation (Slingsby et al., 2021) or activation involving crosstalk with the endothelium. This model could also be used to identify susceptible patient populations at increased risk for thromboembolism by incorporation of cells that are representative of specific patient populations.

4.4 Hepatotoxicity

Effects of ONTs that are dependent on sequence but independent of hybridization often occur in the liver and kidney of nonclinical species. For siRNA, this has been explained as seed region-mediated off-target effects (Janas et al., 2018). Recently, several publications suggested that PS-ASOs can bind manifold intracellular proteins in a sequence- and chemistry-dependent manner (Crooke et al., 2020b). Hydrophobic 2’ ribose modifications like LNA, cEt and 2’F in combination with PS backbone modifications impart higher affinity compared to the same sequence with MOE chemistry. Importantly, highly potent LNA and cEt ASOs can be identified that do not exhibit liver toxicity in vitro or in vivo, but significant screening efforts are required until clear design rules are identified. Hepatotoxicity has been observed with a subset of clinical ASOs and siRNA to date, however, next-generation liver-targeted ONTs appear to mitigate this toxicity somewhat (Tab. 1).

MPS are uniquely positioned to address hepatic liabilities of ONTs during nonclinical development. Two-dimensional hepatocyte models have been a workhorse for high-throughput de-risking of drug-induced liver injury (Sewing et al., 2016). Complex in vitro liver models can enhance long-term hepatocyte survival and tissue-like transcript expression essential to understanding mechanisms of ONT-induced hepatotoxicity (Bell et al., 2018; Messner et al., 2018). Three-dimensional hepatocyte models advantageously prolong hepatocyte phenotype and function over weeks, promote ASGR expression, and enhance target knockdown of GalNAc-conjugates compared to 2D models (Kim et al., 2019b). Identifying possible intracellular RNA hybridization-independent protein targets of PS-ASOs can advance understanding of species sensitivity and translation of nonclinical hepatotoxicity findings to human risk (Crooke et al., 2020b). Rodents are particularly sensitive to GalNAc-conjugated siRNA-mediated hepatotoxicity via siRNA seed region complementarity (Janas et al., 2018). Complex in vitro human liver models that recapitulate the pathophysiology of ONT-mediated hepatotoxicity, potentially involving Kupffer cell activation and inflammatory sequelae (Frazier, 2015), may aid in translating ONT-induced hepatotoxicity findings in nonclinical species to human risk.

4.5 Nephrotoxicity

Owing to their molecular size and charge, ONTs accumulate primarily in the liver and kidney where they can exert both on-target and off-target effects. Renal toxicity has been observed with some clinically approved ONTs and typically manifests in the kidney tubule or glomerulus with differing mechanisms. Renal tubule-related toxicity is distinct from PS-ASO-induced glomerulonephritis related to immunostimulation or complement activation as discussed above. In vitro renal tubule models are warranted to bridge the gap between nonclinical renal toxicity incidence and risk of kidney injury in the clinic. However, nonclinical liver or renal toxicity may not translate to human and, therefore, understanding species-specific mechanisms is important. Preclinical evidence of liver or renal toxicity has not manifested clinically in most cases, perhaps owing to a cautious approach to dosing and clinical trial design. Appreciating that liver and kidney toxicity are general safety liabilities of ONTs, in vitro liver and kidney models may be warranted in nonclinical ONT development regardless of findings in nonclinical species.

Like 2D hepatocyte cultures, 2D proximal tubule epithelial cell cultures are important for toxicity assessment in vitro. For example, an in vitro proximal tubule model established that competitive blocking of receptor-mediated protein endocytosis in the kidney proximal tubule was a putative mechanism of PS-ASO-induced proteinuria (Janssen et al., 2019). Given that shear forces are essential to proximal renal tubule function, MPS incorporating physiologically relevant shear flow may better recapitulate in vivo-like trans-epithelial transport and susceptibility to renal tubule injury (Bajaj et al., 2018). Using human renal proximal tubule epithelial cells (HRPTEC) in 2D and in MPS demonstrated the utility of the MPS model for studying target gene knockdown and following urinary biomarkers during 20 days of culture (Nieskens et al., 2021). Additionally, as mentioned previously, vasculitis and glomerulonephritis are often noted in PS-ASO-mediated kidney injury (Frazier, 2015), thus a kidney-on-a-chip incorporating shear flow, an endothelial lumen, and immune cells would significantly aid assessment of ONT-mediated vasculitis and glomerulonephritis mechanisms in vitro. Physiological relevance must be balanced with throughput, and both 2D and 3D approaches may be warranted at different stages of ONT development to understand and de-risk renal toxicity liabilities prior to first-in-human clinical trials. The value of incorporating kidney MPS into hazard identification of ASOs has recently been demonstrated (Lidberg et al., 2021). For these particular ASOs, the complex nature of on-target, off-target, and chemical toxicity required using human kidney tissue rather than animal models. The authors describe a proximal tubule MPS (commercially acquired) that used a syringe-pump to expose cells (via perfusion) to various ASOs targeting CYP3A45. The authors measured bio-
markers of kidney injury (e.g., kidney injury marker-1 secretion and heme oxygenase-1 induction) and used these data in their overall hazard assessment; the authors concluded that the MPS model was useful for de-risking the potential for nephrotoxicity of their ASOs.

4.6 Influence of route of delivery on ONT-associated toxicity

As mentioned in Section 2, ONT oral bioavailability is limited, and adequate exposure is typically achieved through i.v., s.c., or local administration (Fig. 1). S.c. administration of clinically approved ONTs has been associated with ISRs that had often not been identified nonclinically (Tab. 1). Drisapersen, an investigational ASO indicated for DMD, was submitted for DMD by s.c. administration (Goemans et al., 2018); development was subsequently discontinued. ISRs often exhibit an array of multifactorial clinical manifestations. Their incidence and the failure of nonclinical studies to identify the liability highlight the need to enhance understanding of human susceptibility to ONT-mediated skin toxicity. Given the complex structure and immunological function of the skin, a correspondingly complex human in vitro model is warranted to address ISR liabilities in ONTs. There are multiple in vitro skin toxicity tests that are validated by the Organization for Economic Co-operation and Development (OECD TG 431 (corrosion), 439 (irritation), and 442 (sensitization)(OECD, 2018, 2019, 2021). In vitro skin models have been incorporated into drug development as an efficacy model for inflammatory disease and to study mechanisms of drug-induced hypersensitivity reactions (Hardwick et al., 2020). Although an ISR model is not yet available, full thickness immune-competent skin models may serve to characterize and de-risk drug immunogenicity (Groell et al., 2018), specifically of ONTs employing chemistries known to elicit ISRs.

The first two ONTs approved by FDA were both for ocular indications and administered by intravitreal (i.v.t.) injection. Fomivirsen is a PS-ASO against cytomegalovirus (CMV) IE2 indicated for CMV retinitis, and pegaptanib is a pegylated aptamer against VEGF_{165} indicated for wet age-related macular degeneration (AMD). Serious AEs associated with fomivirsen and pegaptanib are primarily ophthalmological, although rare cases of anaphylaxis have been reported with pegaptanib. Overall, the lack of systemic toxicity by approved ONTs delivered by i.v.t. injection highlights that in patients the blood-retinal barrier restricts entry to systemic circulation. This is not the case for many biologics, as i.v.t.-administered antibodies readily enter the systemic circulation in an Fc domain-dependent manner (Avery et al., 2014). MPS of the blood-retinal barrier may prove useful to gain mechanistic insight of ocular safety and prediction of systemic distribution for i.v.t.-administered ONTs.

Intrathecal (i.t.) administration is required for nusinersen, a PS-ASO indicated for SMA. Nusinersen distributes to CNS tissues and plasma, with half-lives of 4-6 and 2-3 months, respectively, (Biliouris et al., 2018) and has been measured in the liver, skeletal muscle, and kidney (Claborn et al., 2019; 2). Serious adverse events (AEs) associated with nusinersen are systemic in nature (hypotension, TCR, glomerulonephritis, and atelectasis), indicating that i.t.-administered ONTs are not restricted to the CSF. MPS may prove useful to model ONT passage from the CSF to circulation or across the BBB and into the CNS. BBB models may aid elucidation of ADME properties of CNS-targeted ONTs, specifically chemistries influencing BBB penetration, allowing risk assessment of systemic liabilities with i.t.-administered ONTs. Such research would prove invaluable for optimizing next-generation ONTs for neurological diseases and for informing clinical route of administration.

5 Considerations for MPS platform developers

5.1 Productive uptake of ONTs

The term “productive uptake” is often used to describe cellular uptake of ONT that results in the desired pharmacological response. The processes involved in cellular uptake of ONTs are covered in Section 2. To observe PD effect in vitro, cells need the requisite cellular machinery for productive uptake. One way to circumvent limited uptake is through transfection of the ONT (Mancio-Silva et al., 2019). However, transfection bypasses the cellular machinery required for endogenous ONT uptake. Once inside the cell, PD is dependent on, e.g., RNaseH or RISC for ASOs or siRNAs, respectively (Fig. 1). MPS developers should consider whether the cells in their devices can uptake and process ONTs. In these instances, it would be beneficial for MPS developers and ONT manufacturers to collaborate early in model development to ensure proper characterization.

5.2 Promoting and characterizing in vitro phenotypes

A key challenge for MPS, particularly those derived from stem cells, is the promotion and maintenance of tissue-like gene and protein expression. Disease models recapitulating key aspects of the diseased phenotype may enable characterizing the relationship between PD and disease modification, improving the precision of human efficacious dose predictions and safety margins. For toxicity testing, the establishment of a stable, mature tissue-like phenotype may provide appropriate sensitivity to ONT-induced toxicity. However, the promise of MPS for these applications may not be fully appreciated until challenges in deriving mature tissue-like phenotypes can be overcome. Stem or primary cells derived from healthy or diseased patients may harbor the genotype of interest, but considerable optimization of culture conditions may be required to promote the emergence of mature healthy or diseased tissue-like phenotype in vitro. In some cases, primary cells may not be readily available, requiring alternative approaches using embryonic or iPSCs as a starting cell type followed by directed differentiation to the desired phenotype. Often stem cell-directed differentiation falls short of a mature phenotype or may not be feasible or robust enough for stem cell-derived models to be incorporated into drug develop-

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2 https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/209531lbl.pdf
ment pipelines. These challenges require more advancements in basic and applied stem cell biology to be overcome.

Underpinning the necessity for tissue phenotype \textit{in vitro} is the need for quantitative tools to confirm they mimic \textit{in vivo} phenotype. Analyses of phenotype should take advantage of available tools (e.g., qRT-PCR, transcriptomics, ELISA, proteomics) that reliably measure quantitative differences in target gene and protein expression. For disease modeling, robust markers of disease pathology should be measured to allow correlation of PD and functional endpoints. Characterizing ONT-induced toxicity with MPS requires establishing quantitative endpoints \textit{in vitro} that are directly correlated with endpoints (i.e., biomarkers) from animal studies or human pathophysiology to help build confidence in human translation. The use of MPS can also enable characterization of exploratory biomarkers.

5.3 Longevity of culture to observe pharmacology
The selective and durable pharmacology of ONTs makes them an attractive drug class. As shown in Table 1, several approved ONTs are administered in the clinic weekly or less frequently (up to once every three months). Traditional \textit{in vitro} assays are not viable long enough to study the full spectrum of ONT pharmacology. For example, cryopreserved primary human hepatocytes are viable in 2D culture for two or three days, yet the maximal pharmacology may take weeks to develop, and the durability of ONT pharmacology for liver-targeting ONTs is typically weeks to months (Brown et al., 2020). The GalNAc-siRNAs, vutrisiran and inclisiran, exhibit pharmacological activity for up to nine months following a single dose in humans (Habtemariam et al., 2020; Ray et al., 2020). These pharmacological properties of ONTs are preserved in exscheptic tissues as well. For example, nusinersen is administered i.t. as a loading dose followed by maintenance doses once every four months (Tab. 1) enabled by long half-life in the CSF. Inrequent dosing is viewed as a positive aspect for patients and a significant advantage for ONTs as long as adverse on- or off-target events are avoided that would require several weeks or months washout to resolve. To test the durability and potency of ONTs, a robust system is needed whereby target cells and other supportive cells can be maintained for extended periods of time \textit{in vitro or ex vivo}. For example, prolonged culture of human hepatocytes can enable characterization of the efficacy of ONTs and its duration (Yang et al., 2020). \textit{In vitro} hepatocyte models with prolonged duration of activity have been successfully used to evaluate ADME properties of small molecules (Ramsden et al., 2015), but proof of concept data using ONTs is lacking.

For certain contexts of use, MPS may need to be designed with customized media to promote or maintain tissue phenotype for an extended duration. Several tools that may ultimately be useful toward this end include provisional extracellular matrices, optimized ratio of cell culture medium to cells, optimized medium exchange frequency to minimize cost of goods, and, as warranted based on the tissue of interest, the incorporation of perfusion (passive or pump-based) that has been shown to enhance longevity of many cell types \textit{in vitro} (Fig. 2A). Related to the discussion above, non-terminal endpoints for MPS studies of long duration are an essential component of experimental design.

5.4 Bioanalytical considerations
Increased knowledge around the impact of chemical modifications on target transcript hybridization, stability, and conformation and understanding of transcript and protein regulation have resulted in highly potent ONTs requiring very sensitive and reliable bioanalytical tools. Standard bioanalytical techniques applied to the measurement of drug concentration, metabolite profiling, and pharmacology read-out are described in Table 4. Multiple ELISA assays, including double hybridization, and LC-MS/MS and HPLC assays have been validated to support regulatory submissions (Tab. 1). Techniques like stem-loop qPCR and ddPCR have improved assay sensitivity and reduced sample volumes required for quantifying siRNA drug levels (Castellanos-Rizaldos et al., 2020). Further improvements in mass spectrometry methods, including liquid chromatography-coupled high-resolution accuracy mass spectrometry (LC-HRAM), have enhanced sensitivity and throughput (Kim et al., 2019a; Liu et al., 2019). Hybridization LC-fluorescence assays are highly sensitive (LLOQ of 1.0 ng/mL) and can differentiate between parent drug and metabolites but require longer method development for probe design and are therefore restricted to late-stage drug development (Godinho et al., 2017; Tian et al., 2017). Further, high liver tissue quantities (~10 mg) are often needed to characterize drug levels in RISC (Brown et al., 2020) and are not generally attainable \textit{in vitro}. Overall, most of the described bioanalytical tools require high volumes of tissue or plasma, providing a challenge for applying MPS to routine evaluations.

For successful application of MPS in ONT discovery and development, there are general considerations including ability to easily sample effluent and cells, prolonged survival for evaluation of metabolic stability, and metabolite identification.

For RNAi therapeutics, having sufficient material to measure antisense-loaded RISC would be advantageous. This poses a challenge for MPS where cell numbers typically are low (< 20,000) and not easily accessible in the case of microfluidic chips. Evaluation of the metabolite profile requires at least 100 μL effluent and tissue samples using the current mass spectrometry-based tools. A pooled AUC approach might reduce volume/sample needed. Various species in addition to human would allow evaluation of species concordance and/or potential for pharmacologically active or human-specific disproportionate metabolites. For pharmacological assessment, functional and productive uptake into target cells is required. High throughput to evaluate concentration response in target knockdown and longevity to assess recovery would be highly valuable. Toxicological and other endpoint assays would benefit from models where morphology can be readily assessed using light, fluorescence or electron microscopy. Live cell imaging and non-destructive biosensors would enable real-time monitoring of physical and chemical endpoints (Young et al., 2019). One promising area of research is in-line analysis of MPS with biosensors (Fuchs et al., 2021). It has previously been shown that an in-line (or “on-chip”) immu-
obviates the need for end users in the pharmaceutical industry to establish their own research programs, enabling MPS implementation as needed. Ultimately the implementation of MPS in non-clinical development of ONTs will require a collaborative effort by industry, MPS developers, and regulators to establish contexts of use of MPS that address the limitations of traditional animal-based modeling.

6 The utility of MPS to reduce and replace animal use during ONT discovery and development

While MPS are promising tools within the drug development process, they are not currently positioned to replace in vivo animal testing. Based on above considerations, MPS could serve to complement animal studies and ultimately reduce animal usage in nonclinical ONT development. For example, one could envision human in vitro MPS replacing or augmenting early efficacy studies that have been traditionally performed in animals to

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<th>Tab. 4: Current bioanalytical assays</th>
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nosensor had a limit of detection that was one order of magnitude lower than that for an “off-chip” immunoassay (Riahi et al., 2016). As analysts continue to push the limits of detection for these assays, the entire field of MPS will benefit.

5.5 Limitations of MPS

As an in vitro tool, MPS suffers from many of the same limitations as more traditional 2D plate-based models used in screening. Reproducible in vivo tissue-like phenotypes are difficult to derive for many tissues of interest, thus most MPS have focused on liver and kidney. Cost and low throughput are practical limitations of MPS that with time and research can be partially mitigated. Concerns with quality control criteria, reproducibility, and complexity have also hindered their advancement and routine use. Another major hurdle in MPS implementation is the institutional motivation, bandwidth, and funding required to establish and maintain a research program focused on MPS development and implementation. To address this challenge, many MPS developers have established a fee-for-service business model that


understand potency and duration of pharmacologic effect. Additionally, the long duration of the pharmacological effect of ONTs requires a long washout period, precluding re-use of animals which is commonplace for small molecule PK studies with short duration of effect.

Using MPS in early pharmacological and PK assessments could help the later stages of lead optimization and reduce the number of animals used in exploratory studies. Prior to initiating in vivo PK studies, MPS could be employed for metabolite identification, understanding and optimizing active uptake, and characterizing tissue half-life. This could help identify lead compounds by selecting compounds for favorable PK properties without relying on in vivo studies.

Early evaluation in toxicological target organs of interest is an area where MPS could replace animal use, considering toxicological class effects of ONTs are well-characterized. By selecting more robust compounds, the number of dose range-finding studies needed to support pivotal toxicity studies could be reduced. Good Laboratory Practice-compliant pivotal studies in nonclinical animal models are required prior to initiating trials in humans, and it is unlikely that MPS will replace these studies. However, should they arise, MPS could be used to assess mechanisms and help determine whether toxicological findings in the pivotal studies are relevant to humans.

The use and acceptance of MPS in drug development would benefit from having both nonclinical animal and human tissue available in the devices to characterize species concordance. While in vivo animal studies are critical and required to develop any therapeutic, MPS are a promising avenue to replace and reduce animal use throughout ONT development.

7 Discussion and concluding remarks

Oligonucleotide therapeutics have emerged as a novel class of medicines that are distinct from traditional small molecules and from biologics (Fig. 1). As such, new tools are required to fully characterize their behavior and properties. While it may be possible to overcome some of the shortcomings of traditional in vitro tools and in vivo studies, MPS provide a promising technology to recapitulate relevant physiology of human (and potentially nonclinical) animal tissue to evaluate ONTs (Fig. 2). Not only could MPS reduce the number of animals used in drug development, but it could also deliver higher quality human-relevant in vitro data to drive internal and external decision-making. Ideally, MPS designed for evaluating ONTs would contain one or all of the following qualities:

- Permit long-term culture (weeks/months) to match the long tissue half-life and effect duration of ONTs
- Contain suitable number of cells and/or volume of media to conduct bioanalysis
- Be compatible with tissue from relevant species (i.e., human, rat, mouse, dog, monkey) to enable species-specific characterization
- Incorporate tissues from patients or hiPSCs with desired phenotype/genotype

The authors are hopeful that this manuscript will help unite the MPS and ONT communities and encourage continued development of both to ultimately accelerate the pace so that novel ONTs can benefit patients.

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**Conflict of interest**

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