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Improvement of paracellular transport in the Caco-2 drug screening model using protein-engineered substrates

Rebecca L. DiMarco [a, 1], Daniel R. Hunt [b, 1], Ruby E. Dewi [c], Sarah C. Heilshorn [c, *]

[a] Department of Bioengineering, Stanford University, Stanford, CA, USA
[b] Department of Chemical Engineering, Stanford University, Stanford, CA, USA
[c] Department of Materials Science and Engineering, Stanford University, Stanford, CA, USA

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ABSTRACT

The Caco-2 assay has achieved wide popularity among pharmaceutical companies in the past two decades as an *in vitro* method for estimation of *in vivo* oral bioavailability of pharmaceutical compounds during preclinical characterization. Despite its popularity, this assay suffers from a severe underprediction of the transport of drugs which are absorbed paracellularly, that is, which pass through the cell-cell tight junctions of the absorptive cells of the small intestine. Here, we propose that simply replacing the collagen I matrix employed in the standard Caco-2 assay with an engineered matrix, we can control cell morphology and hence regulate the cell-cell junctions that dictate paracellular transport. Specifically, we use a biomimetic engineered extracellular matrix (eECM) that contains modular protein domains derived from two ECM proteins found in the small intestine, fibronectin and elastin. This eECM allows us to independently tune the density of cell-adhesive RGD ligands presented to Caco-2 cells as well as the mechanical stiffness of the eECM. We observe that lower amounts of RGD ligand presentation as well as decreased matrix stiffness results in Caco-2 morphologies that more closely resemble primary small intestinal epithelial cells than Caco-2 cells cultured on collagen. Additionally, these matrices result in Caco-2 monolayers with decreased recruitment of actin to the apical junctional complex and increased expression of claudin-2, a tight junction protein associated with higher paracellular permeability that is highly expressed throughout the small intestine. Consistent with these morphological differences, drugs known to be paracellularly transported *in vivo* exhibited significantly improved transport rates in this modified Caco-2 model. As expected, permeability of transcellularly transported drugs remained unaffected. Thus, we have demonstrated a method of improving the physiological accuracy of the Caco-2 assay that could be readily adopted by pharmaceutical companies without major changes to their current testing protocols.

1. Introduction

Oral drug administration is the most commonly used route because it's safe, convenient, inexpensive, and associated with high patient compliance [1]. In order to be physiologically effective, orally administered drugs must first be absorbed by the body, a process which occurs largely in the small intestines [2,3].

Various cellular assays, including the Caco-2 and Madin-Darby Canine Kidney (MDCK) models, have been developed to serve as *in vitro* models of drug absorption across the healthy small intestinal epithelium [4,5]. The MDCK model suffers from generally low metabolic enzyme activity and transport protein expression [6]. Most notably, MDCK cells greatly under-express P-glycoprotein, a key efflux transport molecule that exists in the plasma membrane of healthy intestinal epithelium and targets drugs to be exported rather than absorbed [7,8]. As such, the most widely used *in vitro* assay to predict the absorption of these drugs is the Caco-2 assay, in which an immortalized human colorectal adenocarcinoma-derived cell line is cultured to confluence on a collagen type-I matrix to model the epithelial lining of the small intestine (Fig. 1a) [9,10]. This assay has been extensively adopted by the pharmaceutical industry due to its ease of use and ability to model the absorption of a variety of compounds [11]. Despite its

* Corresponding author. Materials Science and Engineering Dept, 476 Lomita Mall, McCullough Room 246, Stanford University, Stanford, CA, 94305-4045, USA.
E-mail address: heilshorn@stanford.edu (S.C. Heilshorn).
[1] These authors contributed equally to this work.

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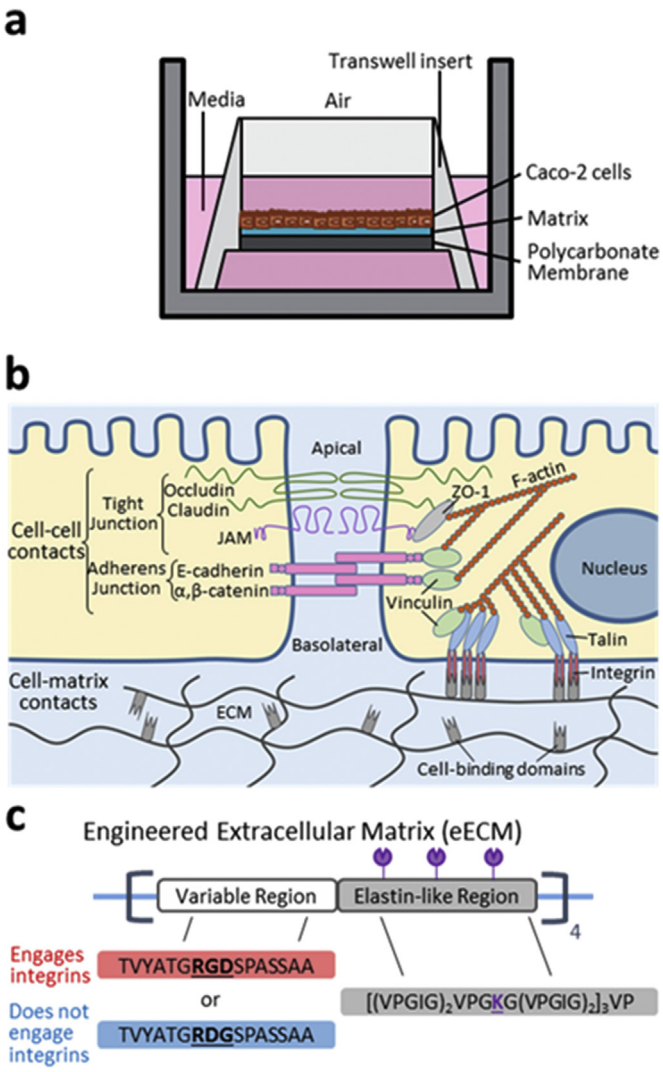

Fig. 1. Schematic of proposed assay to improve paracellular transport for *in vitro* drug screening. (a) Schematic of the Caco-2 monolayer assay in which drug molecules are added to the apical side and their transport to the basolateral side is monitored over time to predict intestinal absorption. (b) Schematic of key cell-cell and cell-matrix interactions that contribute to regulation of paracellular transport through tight junctions that connect neighboring cells. (c) Schematic representation and amino acid sequences of the engineered extracellular matrices (eECMs) used to replace the traditional collagen I matrix in the standard Caco-2 monolayer assay.

prevalence of use, the Caco-2 assay suffers from several shortcomings that limit its physiological accuracy, including atypical mucous production and altered expression of metabolizing enzymes and transport proteins relative to healthy small intestine. Another notable shortcoming of the Caco-2 assay is the significant under-prediction of paracellular absorption, the transport of molecules through tight junctions that connect neighboring cells [10,12,13]. Thus, continued reliance on the Caco-2 assay may result in the rejection of otherwise promising paracellularly transported drug candidates due to artificially poor pharmacokinetic parameters. For example, one of the most commonly prescribed medications worldwide, the paracellularly-absorbed drug ranitidine (Zantac®) was developed before the widespread use of the Caco-2 assay, which incorrectly predicts little to no absorption of this drug [10,14,15].

To address this major limitation, many groups have proposed modified Caco-2 assays to improve its ability to accurately predict *in vivo* biocompatibility via enhanced paracellular transport rate. Typically, these rely on addition of chemicals [16–20] or co-culture with other cell types [12,21–24]. Various three-dimensional (3D) drug screening models have also been developed, including organoid structures derived from primary tissue [25,26] and microfluidic, organ-on-a-chip devices [27]. While scientifically interesting, these strategies are technically cumbersome and not readily translatable to high-throughput settings. Here, we propose an alternative strategy to enhancing Caco-2 paracellular transport by simply modifying the matrix on which the cells are cultured. We hypothesize that modifying cell-matrix contacts will alter the actin cytoskeleton, which will influence cell-cell contacts and hence modulate paracellular transport through intercellular tight junctions. Previous studies have shown that focal adhesions, which can be formed at cell-matrix contact points, influence the development and maintenance of tight junctions through the actin cytoskeleton [28–31]. For example, inhibiting FAK expression or phosphorylation results in decreased barrier function and increased paracellular transport [28]. Additionally, both β1 and β2 subunits of integrins, the transcellular membrane receptors that directly bind to extracellular matrices, have been shown to affect paracellular permeability [32]. Further, disruption of the actin cytoskeleton, known to be regulated by cell-matrix contacts, through the use of small molecule inhibitors of actin polymerization [31] or inhibitors of its upstream effectors myosin light chain kinase (MLCK) [33] and the Rho family of GTP-ases [34,35] has been shown to affect epithelial barrier function.

These data suggest that replacing the standard collagen type I with an alternative, engineered matrix that differentially engages Caco-2 cells is a viable biomaterial approach to modify intercellular tight junctions and regulate paracellular transport. In this work, we utilize in parallel two biomaterials strategies commonly used to influence cell-matrix interactions: modulation of cell-binding domain (CBD) density and tuning of extracellular matrix (ECM) stiffness. Both material properties are known to influence actin cytoskeletal organization and cell spreading [36–39], and we hypothesize that in turn, they will regulate intercellular contacts and paracellular transport (Fig. 1b).

Here, we use a well-characterized and tunable engineered extracellular matrix (eECM) to prove our hypothesis. This eECM is modular in structure, comprising an elastin-like region for control of mechanical properties and, separately, a variable region comprised either of an integrin-engaging sequence or a non-integrin-engaging sequence (Fig. 1c). Expressed as recombinant proteins from genetically encoded plasmids, the eECM's biochemical and biomechanical properties can be tuned independently. With these properties, this eECM allows us to study the effects of CBD density and matrix stiffness, both separately and in combination, on Caco-2 barrier function.

Several methods exist to assess endothelial/epithelial cell monolayer confluence and barrier quality, including determination of fluid filtration coefficient (K_f), hydraulic conductivity (L_p), apparent solute permeability (P_{app}), and transepithelial electrical resistance (TEER) [40,41]. In our work, we have selected to measure the apparent solute permeability (P_{app}) through a drug transport assay, as it is the most direct measurement for evaluating our variable of interest: the permeability of the Caco-2 monolayer to paracellularly transported drugs [42].

We demonstrate that both CBD density and matrix stiffness independently and synergistically affect the paracellular permeability of mature Caco-2 monolayers, with improved permeability observed on cells cultured on matrices of lower CBD density and less mechanical stiffness.