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Transport in Technicolor: Mapping ATP-Binding Cassette Transporters in Sea Urchin Embryos

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SUMMARY

One quarter of eukaryotic genes encode membrane proteins. These include nearly 1,000 transporters that translocate nutrients, signaling molecules, and xenobiotics across membranes. While it is well appreciated that membrane transport is critical for development, the specific roles of many transporters have remained cryptic, in part because of their abundance and the diversity of their substrates. Multidrug resistance ATP-binding cassette (ABC) efflux transporters are one example of cryptic membrane proteins. Although most organisms utilize these ABC transporters during embryonic development, many of these transporters have broad substrate specificity, and their developmental functions remain incompletely understood. Here, we review advances in our understanding of ABC transporters in sea urchin embryos, and methods developed to spatially and temporally map these proteins. These studies reveal that multifunctional transporters are required for signaling, homeostasis, and protection of the embryo, and shed light on how they are integrated into ancestral developmental pathways recapitulated in disease.

"[Multidrug resistance transporters] can be thought of as being more like Swiss army knives than vegetable peelers, with functions dictated by the cellular context in which they are expressed."

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INTRODUCTION

ATP-Binding Cassette Transporters In Development and Disease

Although plasma membrane proteins comprise one quarter of all genes (Almén et al., 2009; Babcock and Li, 2014), our understanding of their functions during development remains limited. This is of interest because the category of plasma membrane proteins includes nearly 1,000 transporters that govern embryo-environment

Abbreviations: ABC, ATP-binding cassette; CAM, calcein-acetoxymethyl ester; CMFDA, chloromethyl fluorescein diacetate; MDR, multidrug resistance; MRP, multidrug-resistance associated protein; SLC, solute carrier.

interactions and intercellular communication within the embryo. Among them are ''active'' transporters that translocate diverse molecules across membranes using power liberated by the direct hydrolysis of ATP. In eukaryotes, the largest group of active transporters is the ATP-binding cassette (ABC) family (Borst and Elferink, 2002); in humans, these include 49 genes divided among seven subfamilies, designated ABC *A-G* (Dean et al., 2001).

Multidrug resistance (MDR) transporters are a subset of ABC transporters that efflux endogenous and exogenous hydrophobic small molecules (Sharom, 2008). These include three subfamilies, the ABCB proteins, including ABCB1/permeabilty-glycoprotein/MDR1 and ABCB4/MDR3; the ABCC/multidrug resistance-associated proteins (MRP), including ABCC1/MRP1, ABCC2/MRP2 and ABCC3/MRP3; and the ABCG proteins, including ABCG2. These transporters can have a dramatic impact on drug disposition (Giacomini et al., 2010) and are often up-regulated in metastatic cancer, leading to chemotherapeutic resistance (Gottesman et al., 2002). Accordingly, these B-, C-, and G- proteins, and several other members of these families, are often designated MDR transporters.

Although MDR transporters have primarily been studied in the context of drug disposition, it is becoming increasingly appreciated that they are also widely expressed in embryos and stem cells (Barbet et al., 2012; Shipp and Hamdoun, 2012; Erdei et al., 2014). By analogy to their drug disposition in adults, one critical function in embryonic cells is presumably protection from xenobiotics. MDR transporters often have large, polyspecific binding sites that accommodate many structurally diverse substrates (Gutmann et al., 2010), including both xenobiotics and signaling molecules. Examples of signaling molecule substrates are platelet-activating factor (Raggers et al., 2001), leukotrienes (Deeley and Cole, 2006), prostaglandins (Russel et al., 2008), and cyclic nucleotides (Cheepala et al., 2013). These signaling molecules have been implicated in many processes of development, but the mechanisms governing their translocation and accumulation are often poorly understood.

Transporter-mediated signaling is emerging as a causative agent in the progression of diseases where transporters are overexpressed (Fletcher et al., 2010). In neuroblastoma, for example, ABCC1 expression is negatively correlated with clinical outcome, even in patients who do not receive chemotherapy, presumably by altering the distribution and/or abundance of endogenous substrates that control cell motility (Fletcher et al., 2010). These observations might suggest that MDR transporters have ancestral functions during development that are related to cell motility and migration, and that these functions become reactivated in disease.

Developmental functions of transporters are further suggested by the observation that pathways common to development and disease, such as the epithelial-mesenchymal transition, can regulate MDR transporters. During embryonic development of triploblastic animals, epithelial cells become mesenchymal through morphological changes, including loss of tight junctions, apical-basal polarity, and cell adhesion; such changes enable individual cells to dissociate from the epithelial layer in which they originate (Thiery et al., 2009). Similarly during metastasis, many types of cancer cells shed epithelial characters, detach from the primary tumor through the epithelial-mesenchymal transition, and become motile (Yang and Weinberg, 2008). These epithelial-mesenchymal transitions can also upregulate MDR-transporter phenotypes in metastatic cancer cells (Arumugam et al., 2009; Saxena et al., 2011). Collectively, such observations suggest that an understanding of the function and regulation of MDR transporters in development would inform our understanding of their behavior in cancer.

ABC Transporters and MDR Transporter Activity In Sea Urchins

MDR transporters are expressed in oocytes, embryos, and stem cells of a variety of model systems, and the list of related plasma membrane proteins found in embryos continues to expand, currently including ABCB4, ABCB5, ABCB11, ABCC2, ABCC3, ABCC4, ABCC5, and ABCC10. Homologs of ABC transporters and MDR-transporter-like efflux activities have been reported in many embryos, perhaps most extensively studied in sea urchins (Good and Kuspa, 2000; Hamdoun et al., 2004; Yabe et al., 2005; Ricardo and Lehmann, 2009; Long et al., 2011; Gökirmak et al., 2012; Fischer et al., 2013; Miranda et al., 2013). While both developmental and protective functions have been proposed for these transporters, relatively few studies have systematically mapped the MDR transporter repertoire of an embryo. Studies on these transporters during the early development of sea urchin embryos, first described nearly a decade ago (Hamdoun et al., 2004), have started to provide insight into the diversity of transporters involved.

Given that marine embryos are exposed to a variety of natural toxins and anthropogenic contaminants (Epel et al., 2008), one function of sea urchin MDR transporter activity appears to be protecting embryos from xenobiotics. This was suggested by the fact that inhibitors of MDR transporters sensitize embryos to environmental toxicants (Bosnjak et al., 2009) as well as chemotherapy drugs such as vinblastine (Hamdoun et al., 2004; De Souza et al., 2010; Anselmo et al., 2012) and etoposide (Epel et al., 2006). Pollutants such as mercuric chloride (Bosnjak et al., 2009) and antifouling agents (Bosnjak et al., 2011; Xu et al., 2011) were also shown to be substrates of MDR transporters. In addition to protecting embryos from chemical insults, transporters protect against ultraviolet radiation, albeit the mechanism remains unknown (Leite et al., 2014).

Additional insights into the functions of sea urchin MDR transporters come from genomics. The purple sea urchin (*Strongylocentrotus purpuratus)* genome currently has 82 reliable ABC transporter gene annotations (EchinoBase.org; Goldstone et al., 2006; Sodergren et al., 2006). At least 75 of the 82 genes are expressed in early larval stages, highlighting their importance by development. The expansion in sea urchin ABC genes, as compared to humans

(Vasiliou et al., 2009), is primarily in the MDR transporter families B, C, and G –including 13 ABCB genes, 31 ABCC genes, and 9 ABCG genes. At least 20 B, C, and G genes are expressed by gastrulation (Shipp and Hamdoun, 2012) and the corresponding proteins encoded by these 20 genes are predicted to function in diverse processes including cell signaling, lysosomal and mitochondrial homeostasis, potassium channel regulation, pigmentation, and protection from xenobiotics (Shipp and Hamdoun, 2012).

Insights into the regulation and developmental significance of these proteins have come from studies revealing that B- and C-type efflux transport is dynamic in early development (Fig. 1A). For example, there is an 80-fold increase in MDR transporter activity that occurs 25 min

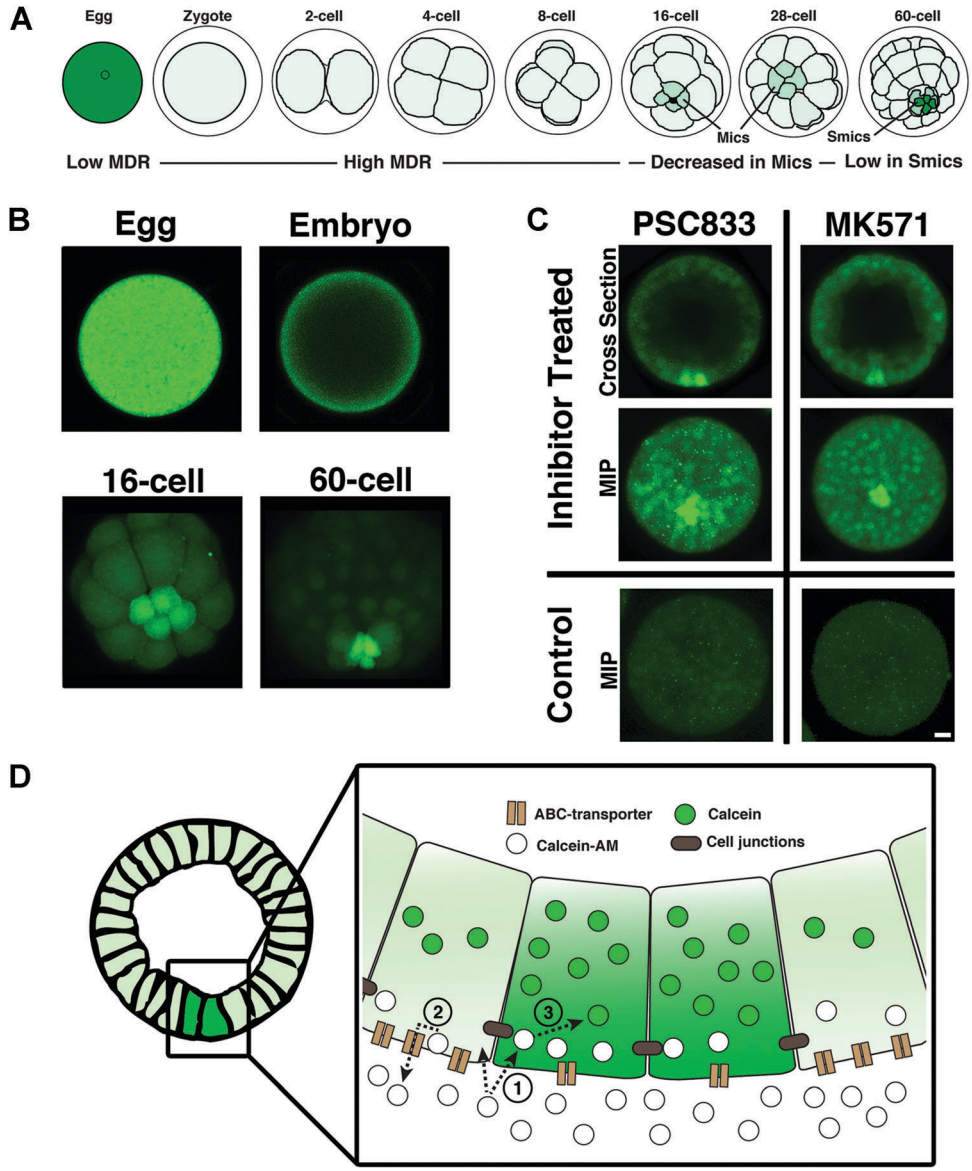

Figure 1. Transitions in MDR transporter activity during development. **A**: Schematic illustration of switches in MDR transporter activity during sea urchin development. Efflux activity increases 25 min after fertilization (cell color transitioning from dark to light green) and later decreases in small micromeres of 60-cell embryos (light to dark green), as demonstrated by changes in calcein accumulation. **B**: Confocal micrographs show differences in calcein accumulation (green) after the egg-to-embryo and soma-to-germ line (a.k.a. micromere-to-small micromere) transition. **C**: Confocal maximum intensity projections (MIP) and cross-sections showing calcein accumulation after treatment with 10 μM PSC833 (MDR-transporter inhibitor) or 10 μM MK-571 (MRP inhibitor) in 16-hour-old blastulae. All embryos were imaged and displayed with the same settings, but brightness of control embryos was increased in this figure to make them apparent. **D**: Schematic of a calcein-AM efflux assay in polarized epithelial cells of the blastula. (1) Calcein-AM passes into/through the plasma membrane where it is (2) recognized and effluxed by an ABC transporter or (3) cleaved into the fluorescent product, calcein, by intracellular esterases. Calcein is fluorescent and membrane impermeable, and can therefore accumulate in the cytoplasm.

after fertilization (Fig. 1B). This increase in activity does not require de novo gene expression, but is instead mediated by actin-dependent translocation of transporters stored in the unfertilized eggs to the tips of microvilli in zygotes, via a Rab11-dependent mechanism (Hamdoun et al., 2004; Whalen et al., 2012). Such regulation of transporter activity is analogous to that reported in mammalian adult hepatocytes, where bile acid secretion is regulated by serine/threonine kinases (LKB1 and AMPK1) (Fu et al., 2011; Homolya et al., 2014) and trafficking of ABCB11 from Rab11 and myosin Vb-positive vesicles to the apical surface (Wakabayashi et al., 2006).

Of interest is whether the LKB1-AMPK1 pathway, or other kinase signaling pathways, might also control transporter activity in embryos. LKB1-AMPK1 signaling is involved in developmental programs and in various disease phenotypes. For instance, in Lkb1-knockout mouse embryos, neural tube closure, somitogenesis, and vascular development are defective (Ylikorkala et al., 2001; Londesborough et al., 2008). In disease, LKB1 is a tumor repressor and LKB1 knockdown causes epithelial-mesenchymal transition in lung carcinogenesis (Roy et al., 2010), suggesting that the LKB1-AMPK1 signaling pathway may be involved in epithelial-mesenchymal-transition-induced multidrug resistance.

In sea urchin embryos, MDR transporter activity remains high in early development, but is down-regulated only in small micromeres, the presumptive germ-line progenitors (Fig. 1B and C). This is unexpected given that high transport activity is seen is some stem cells, although a likely possibility is that modulation of transporter activity is necessary for primordial-germ-cell functions such as migration (Campanale and Hamdoun, 2012; Campanale et al., 2014). Another developmentally regulated ABC transporter is ABCC5a, which is expressed only transiently during development (Shipp and Hamdoun, 2012). Though the precise developmental function of ABCC5 remains unknown, a recent report suggested that ABCC5 has a conserved role in heme homeostasis and hematopoiesis (Korolnek et al., 2014). Interestingly, sea urchin ABCC5a is spatially restricted to newly forming secondary mesenchyme, the precursors of larval immunocytes (Shipp and Hamdoun, 2012; Solek et al., 2013).

Given the diversity of transporters utilized by embryos, the proposed roles for ABC transporters are clearly the ''tip of the iceberg''. Here, we summarize approaches for mapping MDR transporters in sea urchin embryos, and the implications of these studies for understanding transporter function. We review methods for measuring efflux activities using fluorescent substrates, expression of recombinant proteins, and mapping of transporter localization. Although we focus on MDR transporters, which we define as plasma membrane proteins in the B, C, and G families, the techniques are also applicable to the study of other types of membrane proteins, such as solute carrier (SLC) transporters (Wu et al., 2011b) and amino acid transporters (Meyer and Manahan, 2009), and they provide a road map for the study of membrane transporters in other embryo models (Fischer et al., 2013).

FROM GENE TO FUNCTION: EFFLUX ASSAYS, EXPRESSION OF RECOMBINANT TRANSPORTERS, AND MAPPING TRANSPORT WITHIN THE EMBRYO

Use of Efflux Assays to Study MDR Transporter Function

Efflux assays with fluorescent substrates were first developed to assess MDR transporter function in drug-resistant cancer cells (Homolya et al., 1993; Homolya et al., 1996). Since then, they have been adapted for applications ranging from drug discovery (Polli et al., 2001; Tegos et al., 2014) to pollutant testing (Hamdoun et al., 2002; Smital et al., 2004; Bosnjak et al., 2009; Xu et al., 2011) to developmental studies (Hamdoun et al., 2004; Campanale and Hamdoun, 2012; Whalen et al., 2012). MDR transporter activity is assessed by measuring the accumulation of fluorescent transporter substrates (Table 1). If transporter activity is high, fluorescent substrates are effluxed and intracellular fluorescence is low; conversely, low transporter activity allows fluorescent substrates to accumulate, leading to high intracellular fluorescence (Figure 1).

Fluorescent efflux assays can be performed with different detection tools, such as a spectrofluorometer (Cole et al., 2013) or microscope (Campanale and Hamdoun, 2012; Gökirmak et al., 2012). Our group has favored the use of confocal microscopy since this method can be used for quantitative and/or qualitative measurement of transport. As compared to spectrophotometry of homogenates or widefield microscopy, confocal microscopy further enables the measurement of differences in efflux between cell types of the embryo (Campanale and Hamdoun, 2012).

Fluorescent Substrates of MDR Transporters

Due to their polyspecificity, MDR transporters can efflux a wide variety of structurally diverse compounds. Conveniently, these include many fluorescent small molecules ($<$1000 Da), which can be readily visualized in efflux assays (Litman et al., 2000; Lebedeva et al., 2011; Strouse et al., 2013). Interactions between mammalian MDR transporters and fluorescent substrates are well characterized, allowing the use of this approach to understand the efflux functions of embryo transporters. Functional characterization of sea urchin MDR transporters with these molecules, showed that they have similar efflux activities to their closest human homologs (Gökirmak et al., 2012).

Fluorescent substrates can be grouped into four major categories (Table 1). The first category contains the fluorone-based synthetic compounds, including rhodamines (e.g. rhodamine 123, rhodamine B, and rhodamine 6G); calcein-acetoxymethyl ester (CAM); 2′, 7′-bis-(2-Carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM); Fluo3-AM; fluorescein diacetate (FDA); and chloromethyl fluorescein diacetate (CMFDA). Among them, CAM has been the probe most commonly used in echinoderm eggs and embryos (Hamdoun et al., 2004; Roepke et al., 2006; Campanale and Hamdoun, 2012; Gökirmak et al., 2012). CAM is a neutral, non-fluorescent, membrane-permeable substrate of ABCB- and ABCC-type

TABLE 1. Fluorescent Substrates of MDR Transporters

Dye Class	Fluorescent Dye	Target MDR Transporter			References
		ABCB1	ABCC1	ABCG2	
Fluorone	Rhodamine 123	+	−	+	Litman et al. (2000); Szakács et al. (2008)
	Calcein-AM/calcein	+	+	−	Litman et al. (2000); Gökirmak et al. (2012)
	BCECF-AM/BCECF	+[a]	+[a]	−	Draper et al. (1997); Homolya et al. (1993)
	Fluo3-AM	N/D	+	N/D	Keppler et al. (1999)
	CMFDA	+[a]	+[a]	N/D	Weiss et al. (2007)
	FDA	+[a]	+[a]	N/D	McAleer et al. (1999)
Bodipy	Vinblastine	+	+	−	Litman et al. (2000); Gökirmak et al. (2012)
	Verapamil	+	−	−	Litman et al. (2000); Gökirmak et al. (2012)
	Paclitaxel	+	−	−	Litman et al. (2000)
	Prazosin	+	−	+	Litman et al. (2000)
Cyanine	JC-1	+	+	+	Strouse et al. (2013)
	Mitotracker Green	+[a]	+	−	Marques-Santos et al. (2003); Strouse et al. (2013)
	SYTO stains	+	+	−	Strouse et al. (2013)
Anthracene	Doxorubicin	+	+	+	Litman et al. (2000); Szakács et al. (2006)
	Daunorubicin	+	+	+	Litman et al. (2000); Szakács et al. (2006)
	Bisantrene	+	−	+	Zhang et al. (1994); Litman et al. (2000)
	Epirubicin	+	+	+	Szakács et al. (2006)
	Mitoxantrone	+/−	+	+	Litman et al. (2000); Gökirmak et al. (2012)

+ verified substrate; − absence of transporter-substrate interaction; +/− interactions differ among species; N/D, not determined.
[a]Unpublished observation in sea urchin embryos.

transporters in mammals and sea urchins. CAM passively diffuses across the cell membrane, and is converted to membrane-impermeable green fluorescent calcein after the cleavage of the acetoxymethyl ester (-AM) moiety by intracellular esterases (Fig. 1D) (Essodaigui et al., 1998). Therefore, cells with high ABCB and ABCC MDR transporter activity accumulate less calcein and exhibit lower fluorescence compared to cells with less activity. While fluorone-based dyes are typically not effective substrates of ABCG transporters, a related class of xanthene-based dyes are alternative substrates for these proteins (Lebedeva et al., 2011).

The second major group of fluorescent compounds includes the bodipy conjugates of MDR transporter substrates, including vinblastine, verapamil, paclitaxel, and prazosin (Litman et al., 2000). These substrates have no inherent fluorescence, but bodipy is strongly fluorescent and does not require esterase activity for activation. Bodipy is relatively nonpolar and neutral, which limits, but does not eliminate, effects on the substrates to which it is conjugated − thus, an important consideration when using these substrates as tracers in that the bodipy moiety can slightly alter the characteristics of the substrate to which it is attached. For example, bodipy-verapamil is more effectively transported by ABCB1 than unconjugated verapamil, and is also a less effective inhibitor (Lelong et al., 1991). Regarding specificity, bodipy conjugates of vinblastine and verapamil are well-characterized substrates for mammalian ABCB transporters (Litman et al., 2000; Crivellato et al., 2002; Kimchi-Sarfaty et al., 2002), and both are effluxed by sea urchin permeability-glycoprotein-type transporters such as ABCB1a and ABCB4a (Gökirmak et al., 2012). These observations illustrate the conservation of substrates between species.

The third class of fluorescent substrates includes cyanine-based fluorescent compounds such as JC-1 (a mitochondrial membrane potential probe), MitoTracker Green FM (a mitochondrial stain), and the SYTO series of nucleic acid stains. JC-1 is effluxed by all three major MDR transporters, and has been used in combination with niclosamide to probe ABCG2 activity (Strouse et al., 2013). MitoTracker Green FM is a substrate of both human and sea urchin ABCB type transporters (Marques-Santos et al., 2003; unpublished observations).

Finally, the fourth class of fluorescent MDR-transporter substrates contains the anthracene-derived antitumor drugs. These include doxorubicin, daunorubicin, bisantrene, epirubicin, and mitoxantrone (Litman et al., 2000). As they are inherently fluorescent, these drugs can be used as tools for studying efflux functions of MDR transporters in intact cells, but their cytotoxicity and comparatively low fluorescence make them difficult to use at optimal concentrations. In some cases, they remain the best available option. For example, mitoxantrone is effluxed by human ABCG2, and it is also a weak substrate for human ABCB1 (Litman et al., 2000; Sharom, 2008). In sea urchins, mitoxantrone is also effluxed by ABCG2a, but we were unable to detect significant efflux of this substrate by ABCB1a or ABCB4a (Gökirmak et al., 2012).

When using fluorescent-efflux assays to characterize MDR transporters, one limitation is the inability to resolve activities of individual transporters, as many MDR transporters display overlapping substrate specificity. For example, in humans, CMFDA can be effluxed by ABCC1, ABCC2, ABCC3, and ABCC5 transporters (McAleer et al., 1999; Weiss et al., 2007). In addition, some of the dyes used as substrates of MDR transporters are also substrates of SLC transporters. For example, Fluo-3 is transported both

TABLE 2. Inhibitors of MDR Transporters

Inhibitor Class	Inhibitor	Target MDR Transporter		References
		Primary	Secondary	
First Generation	Verapamil	ABCB1	ABCC1/ABCG2	Tsuruo et al. (1981); Germann et al. (1997); Anselmo et al. (2012) [a]
	Cyclosporin A	ABCB1	ABCC1/ABCG2	Qadir et al. (2005); Matsson et al. (2009); Hamdoun et al. (2004) [a]
	Amiodarone	ABCB1	ABCG2	Ford and Hait (1990); Matsson et al. (2009)
	Quinidine	ABCB1	ABCC1	Robert and Jarry (2003); Hamilton et al. (2001); Matsson et al. (2009)
	Nifedipine	ABCB1	ABCG2	Philip et al. (1992); Zhang et al. (2005)
Second and third Generation	R-Verapamil	ABCB1/ABCC1		Perrotton et al. (2007)
	PSC 833	ABCB1	ABCC1	Twentyman (1992); Leier et al. (1994); Campanale and Hamdoun (2012) [a]
	Elacridar	ABCB1/ABCG2		Hyafil et al. (1993); de Bruin et al. (1999); Oostendorp et al. (2009)
	Tariquidar	ABCB1	ABCG2	Martin et al. (1999); Robey et al. (2004); Kannan et al. (2011)
	LY465803	ABCC1	ABCB1	Dantzig et al. (2004); Norman et al. (2005)
	LY475776	ABCC1	ABCB1	Dantzig et al. (2004)
Fourth Generation	Fumitremorgin C	ABCG2		Rabindran et al. (2000); Matsson et al. (2007); Matsson et al. (2009)
	Ko143	ABCG2	ABCB1	Allen et al. (2002); Matsson et al. (2009)
	Curcumin	ABCB1/ABCC1/ABCG2		Wu et al. (2011a); Limtrakul et al. (2007)
Others	MK-571	ABCC1	ABCB1/ABCG2	Leier et al. (1994); Matsson et al. (2009); Fischer et al. (2013) [a]
	BAY u9773	ABCC1		Maeno et al. (2009)

[a]References that show validation in embryos.

by inwardly directed SLC and outwardly directed ABC transporters (Sai and Tsuji, 2004; Baldes et al., 2006). One solution to this problem can be found by combining the use of fluorescent dyes with specific inhibitors. Indeed, a recent screen of 121 fluorescent compounds in multidrug-resistant human cell lines identified 31 substrates, which can be used in combination with inhibitors to specifically probe ABCB1, ABCC1, and ABCG2 transporter activities (Strouse et al., 2013).

Inhibitors of MDR Transporters

The efflux activity measured in embryos can occur from the action of multiple, redundant transporters. In sea urchins, for example, CAM is a substrate of ABCB1a, ABCB4a, and ABCC1β, a splice variant of ABCC1 (Gökirmak et al., 2012; unpublished observations). Identifying the proportional contributions of individual transporters to the global efflux thus requires specific ABC transporter inhibitors. MDR-transporter inhibitors are often small molecules that were initially generated to improve drug retention in tumor cells. Attempts to discover such molecules have resulted in the development of preclinical and clinical drugs, some of which are useful for characterizing the efflux functions and structures of MDR transporters in vivo and in vitro. Most inhibitors target the major MDR transporters –such as ABCB1, ABCC1, ABCC2, and ABCG2– although, it is important to note that many of these compounds also inhibit other ABC transporters at higher concentrations (Table 2).

There have been four ''generations'' of MDR transporter inhibitors, each with successively greater levels of specificity for MDR transporters. First-generation inhibitors were derived from compounds with known biological functions, such as channel blockers (e.g. verapamil and nifedipine), immunosuppressants (e.g. cyclosporine), and cardiovascular drugs (e.g. amiodarone and quinidine). Since MDR transporters are often not the only targets of these compounds, they yielded limited clinical success due to their undesirable toxicity. For instance, verapamil, an L-type calcium channel blocker, sensitizes multidrug-resistant leukemia cells (Tsuruo et al., 1981) and later was shown to be a competitive inhibitor of ABCB1 (Yusa and Tsuruo, 1989); however, verapamil is cardiotoxic at the concentration that inhibits ABCB1 (Krishna and Mayer, 2000). In echinoderm embryos, verapamil and cyclosporine A were used to characterize ABCB activity (Hamdoun et al., 2004; De Souza et al., 2010; Anselmo et al., 2012). As with mammalian systems, the inhibition of sea urchin MDR transporters requires low micromolar concentrations of both compounds, whereas relatively high concentrations of verapamil are required to inhibit calcium channels (Kazazoglou et al., 1985).

Second- and third-generation MDR-transporter inhibitors were designed to address some of these targeting problems. Compounds of these generations include PSC833 (a non-immunosuppressant cyclosporine D analog), the R-enantiomer of verapamil (a lower affinity calcium channel antagonist), and anthranilic acid derivatives such as tariquidar and elacridar, which are potent inhibitors of

ABCB1 and ABCG2 transporters (Hyafil et al., 1993; de Bruin et al., 1999; Martin et al., 1999; Robey et al., 2004). Recently, a fluorophore conjugate of a tetrazole-containing analog of tariquidar, HM30181, was developed for real-time imaging of MDR transporter-inhibitor interactions (Sprachman et al., 2014). Among second- and third-generation inhibitors, PSC833 has been used in sea urchin embryos to study ABCB-type transporters (Fig. 1C; Table 2), although one of its limitations is poor solubility at high concentrations ($>10\,\mu M$).

Fourth-generation MDR-transporter inhibitors include compounds discovered through screens from natural products extracted from plants, fungi, and marine organisms. This family of inhibitors consists of structurally diverse compounds that can be used as scaffolds for de novo synthesis and the design of new inhibitors (Wu et al., 2011a). For example, fumitromorgin C (FTC) is a highly specific ABCG2 inhibitor isolated from fungi (Rabindran et al., 2000), but its undesirable neurotoxicity leaves it unusable in medicine. Ko143, a structural analog of FTC, on the other hand, is not neurotoxic and is highly specific to ABCG2 (Allen et al., 2002).

Finally, although most of the initial efforts to reverse MDR transporter phenotypes focused on ABCB1, several compounds were found to specifically inhibit ABCC1 and ABCC2 (Table 2). Among those, MK-571, a leukotriene (LT) D4 analog, is commonly used (Cole, 2014). Although it was originally developed to inhibit cysteinyl leukotriene receptor 1 (CysLTR1) (Young, 1991), later reports show that it competitively inhibits ABCC1-mediated efflux of LTC_4 (Leier et al., 1994) and proved to be non-selective among MRP homologs, including ABCC2, ABCC3, ABCC4, and ABCC5 (Haimeur et al., 2004). Furthermore MK-571 can inhibit organic anion transporters at high concentrations (Keppler, 2011).

Other CysLTR1 antagonists, including ONO-1078, LY171883, and the dual CysLTR1/2 antagonist BAY u9773, were shown to competitively inhibit ABCC1 transporter activity, but the cross-inhibition of MRP homologs by these compounds still remains an issue (Cole, 2014). To overcome specificity issues, cyclohexyl-linked tricyclic isoxasole inhibitors were developed for ABCC1 transporters. LY465803 and its photoactive analog LY475776 are very potent competitive inhibitors of ABCC1 ($IC_{50} \sim 50$ nM), and unlike MK-571, they do not inhibit the closely related transporter ABCC2 or other MRP homologs (Dantzig et al., 2004; Norman et al., 2005). Among these MRP inhibitors, only MK-571 (Fig. 1C) has been used in sea urchin embryos to date (Hamdoun et al., 2004; Epel et al., 2006; Bosnjak et al., 2009). Within sea urchins, it has proven to be an effective inhibitor of CAM efflux, although this could be due to its action on multiple transporters; therefore, it will be important to determine if alternative ABCC inhibitors are also effective and specific in sea urchins.

Linking Transporters to Substrates through the Expression of Recombinant Proteins

Efflux assays often fall short of linking a specific transporter to an observed activity due to the overlap in substrates among transporters. In sea urchins, a simple method to address this is the expression of a recombinant transporter encoded by an exogenous mRNA that is injected into the one-cell embryo. Exogenous mRNA expression is a routine procedure used to study gene function in sea urchins (Cheers and Ettensohn, 2004), frogs (Churamani et al., 2012), zebrafish (Postel et al., 2011), amphioxus (Holland and Yu, 2004), starlet sea anemones (Layden et al., 2013), and fruit flies (Beumer et al., 2008). We recently adapted such methods for fluorescent protein tagging and expression of ABC transporters in sea urchins (Gökirmak et al., 2012).

Given the sensitivity of transporters to the location of fluorescent protein tags, we generated pCS2 variants, termed pCS2 + 8, that facilitate the construction of untagged, as well as N- and C-terminal transporter fusions in a common vector backbone (Fig. 2) (Addgene.org; Gökirmak et al., 2012). The resulting mRNAs are injected into a one-cell embryo, which is then cultured to allow sufficient time for expression. We often assess efflux on early blastulae (12–20 hr post-fertilization) (Fig. 2) since the embryos are still immobile at this stage, and consist of a single layer of polarized cells (Itza and Mozingo, 2005). By this time, more than 90% of the observed efflux activity can come from the recombinant protein, thus providing a system in which to test subtle differences in functions of homologs, paralogs, mutants or splice variants. For example, we found that two amino acids in transmembrane helix (TMH) 6 are responsible for differences in stereoselectivity of sea urchin versus mouse ABCB1a (Gökirmak et al., 2012).

Recombinant protein expression informs also provides information regarding the subcellular localization of a specific transporter. For example, Figure 3 shows recombinant versions of 12 ABC transporters representing B-, C-, and G-subfamilies. Of these, five, –ABCB1a, ABCB4a, ABCC1β (a splice variant of ABCC1), ABCC5a, and ABCG2a–are plasma membrane transporters with a clear efflux activity for fluorescent substrates (Table 1) (Gökirmak et al., 2012). Conversely, two basolateral transporters, ABCB1b and ABCC4, and an apical transporter, ABCG2c, do not appear to have activity against any of the substrates we have tested to date. Four half transporters –ABCB6, ABCB7, ABCB8, and ABCC9a (SUR2 homolog)– have organellar membrane localizations (Fig. 3), consistent with the residence of their closest mammalian homologs.

Modeling Transporter Location Within the Embryo

Merging physiological studies with developmental "mapping" approaches (quantitative PCR and in situ hybridization) can further refine our membrane localization models in sea urchins (Fig. 4), which implicate spatial and temporal restriction of transporters with specific morphogenetic or protective functions. For example, we found that the predicted xenobiotic transporter ABCB1a is expressed at high levels and is present in most cells of the embryo throughout the first three days of development.

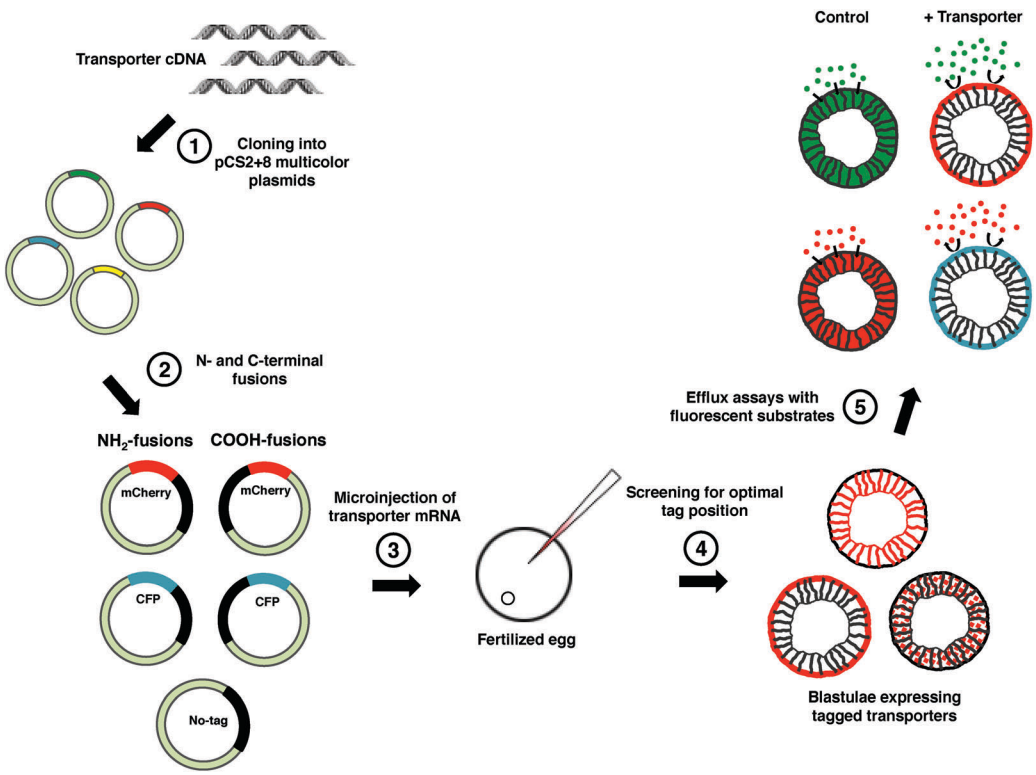

Figure 2. Mapping subcellular location and determining substrate specificity of sea urchin ABC transporters. **1**: Full-length coding sequences of ABC transporters are cloned into pCS2 + 8 multicolor plasmids. **2**: N- and C-terminal fluorescent protein fusions or untagged variants of each ABC transporter are generated to test the positional effect of the fluorescent protein tag on protein localization and expression. **3**: Synthetic ABC transporter mRNA is synthesized in vitro from linearized plasmid, and is then microinjected into fertilized embryos. **4**: Subcellular localizations and (5) efflux activities of each tagged ABC transporter are determined in blastulae using confocal microscopy.

In contrast, ABCC5a, which is similar to a human homolog implicated in signaling, is expressed in a spatially and temporally restricted fashion that is consistent with a function in developmental signaling (Shipp and Hamdoun, 2012). Activity and localization assays indicated that ABCB1a is a polyspecific, apical transporter (i.e. facing the environment), whereas ABCC5a is basolateral (facing neighboring cells and the blastocoel) and has minimal xenobiotic efflux activity (Gökirmak et al., 2012; Shipp and Hamdoun, 2012). Together, these recombinant protein and gene expression studies can be used to model plasma membrane protein localization during development (Fig. 4).

Future Approaches to Link Specific Substrates to Developmentally Relevant Transporters

One future direction will be to connect what we are learning about the spatial and temporal distribution of transporters in embryos to an understanding of their substrates. While MDR transporters efflux many signaling molecules important for reproduction and development, few fluorescent analogs of these compounds are available. Vesicular transport studies with overexpressed proteins are an important alternative approach for studying the translocation of such transporters (Horio et al., 1988). Indeed, several membrane systems —including insect cells (Bakos et al., 1998; Fischer et al., 2013), transfected or selected mammalian cell lines (Zeng et al., 2000), artificial membrane vesicles (Sharom et al., 1999), or monolayer transport assays (Polli et al., 2001)—have previously been used to directly measure the translocation of a radioactively labeled substrate across a cell membrane. Since active transport of substrates across cell membranes by ABC transporters requires hydrolysis of ATP molecules and the release of inorganic phosphate (P_i) and ADP, ATPase assays with purified transporters can also be used to measure the rate of P_i liberation after stimulation with a presumed developmental substrate (Baykov et al., 1988; Henkel et al., 1988). It is generally accepted that compounds that stimulate ATPase activity are substrates, while those that inhibit ATPase activity are inhibitors (Al-Shawi et al., 2003). Additionally, vesicular transport assays have recently been combined with liquid chromatography/mass spectrometry (LC/MS)-based metabolomics to identify physiological MDR transporter substrates (Krumpochova et al., 2012).

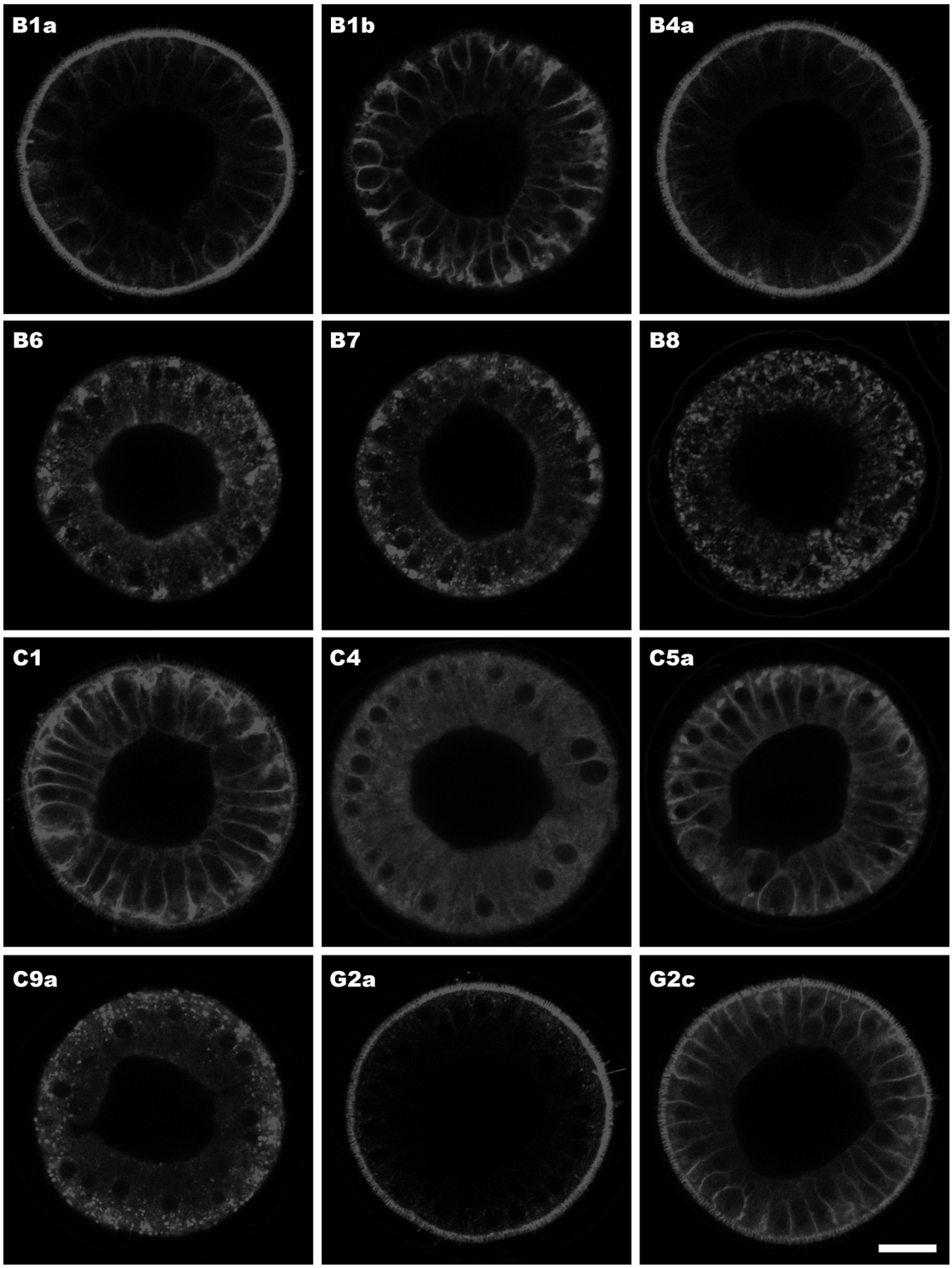

Figure 3. Subcellular localization of mCherry-tagged sea urchin ABC transporters (*Sp*-ABC) in blastulae. ABC-B1a, B4a, G2a, and G2c localize to apical membranes in *S. purpuratus* embryos. ABC-B1b, C1, C4, and C5a localize to basolateral membranes. ABC-B6, B7, B8, and C9a localize into internal/organellar membranes. Scale bar, 20 μm.

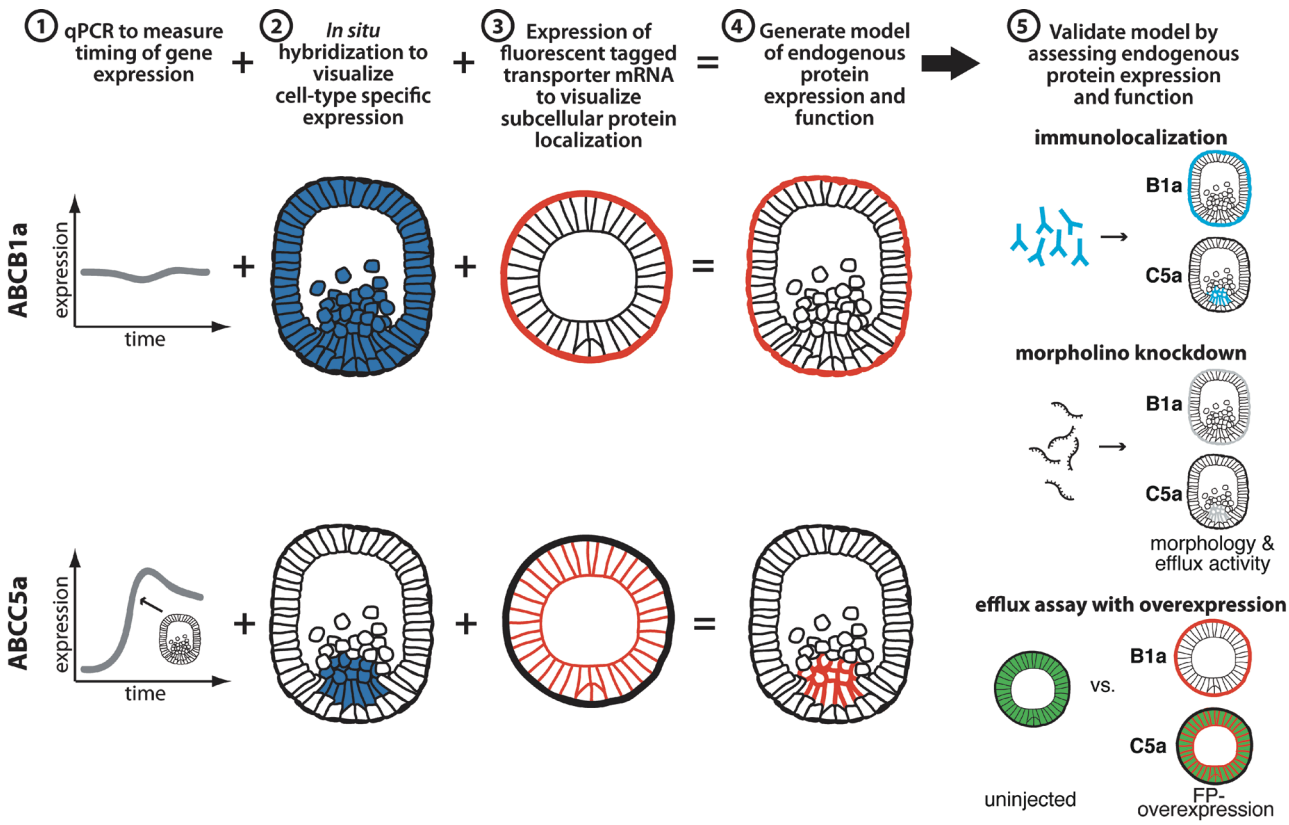

Figure 4. Spatio-temporal mapping of transporters. **1**: Temporal expression patterns of transporters reveal developmental stages in which transporters are expressed. For example, ABCB1a expression is not restricted to a specific developmental stage, while ABCC5a expression is primarily expressed at and after the mesenchyme blastula stage. **2**: Spatial patterns of transporter expression are determined by in situ hybridization. ABCB1a is expressed in all cells of the embryo, while ABCC5a is only expressed in non-skeletogenic mesenchyme cells (blue). **3**: Subcellular localization of relevant proteins are determined by the expression of fluorescent protein fusions of a transporter (red) (see also Figs. 2 and 3). For example, ABCB1a localizes to the apical membrane while ABCC5a localizes to basolateral membranes. **4**: Data are merged to model endogenous transporter protein expression and function. **5**: Models are tested by assessing endogenous protein expression, developmental function, and xenobiotic efflux activity.

CONCLUSIONS

Despite the significance of membrane transport, our understanding of plasma membrane transporters in embryos remains rudimentary. While membrane transport has long been studied in adult cells, such as hepatocytes or in cancer cells, there are several challenges to applying this existing information to embryos. First, unlike membrane transport in differentiated cell models, such as adipocytes, neurons, hepatocytes, or renal cells, the surfaces of embryonic cells are dynamic, and transporter composition changes rapidly with differentiation. Second, the number of transporters is large, and transport is often mediated by the simultaneous action of multiple, overlapping/redundant transporters (Giacomini et al., 2010). Finally, unlike most enzymes, membrane transporters are polyspecific, i.e., have multiple substrates. Thus, MDR transporters can be thought of as being more like Swiss army knives than vegetable peelers, with functions dictated by the cellular context in which they are expressed.

Nonetheless, the challenges underlying the identification of embryonic membrane transporter function are essential to tackle for several reasons. The first is simply that the functions of many membrane transporters are incompletely understood in any system. As illustrated here, understanding how these proteins are regulated in space and time in an embryo can be an important tool for generating hypotheses about their potential functions. This is especially relevant given that the expression of membrane transporters in diseases such as cancer can itself result from recapitulation of developmental pathways, including the epithelial-to-mesenchymal transformation pathways. Further, as alluded to above, the actual function of those transporters in disease can be analogous to their developmental roles, such as controlling cell motility. Thus, coming to grips with the diversity of functions and regulatory pathways that these Swiss army knives participate in may require insight from embryos.

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