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ATP-Binding Cassette (ABC) Transporter Expression and Localization in Sea Urchin Development

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Background: ATP-binding cassette (ABC) transporters are membrane proteins that regulate intracellular concentrations of myriad compounds and ions. There are >100 ABC transporter predictions in the *Strongylocentrotus purpuratus* genome, including 40 annotated ABCB, ABCC, and ABCG "multidrug efflux" transporters. Despite the importance of multidrug transporters for protection and signaling, their expression patterns have not been characterized in deuterostome embryos. Results: Sea urchin embryos expressed 20 *ABCB*, *ABCC*, and *ABCG* transporter genes in the first 58 hr of development, from unfertilized egg to early prism. We quantified transcripts of *ABCB1a*, *ABCB4a*, *ABCC1*, *ABCC5a*, *ABCC9a*, and *ABCG2b*, and found that *ABCB1a* mRNA was 10–100 times more abundant than other transporter mRNAs. In situ hybridization showed *ABCB1a* was expressed ubiquitously in embryos, while *ABCC5a* was restricted to secondary mesenchyme cells and their precursors. Fluorescent protein fusions showed localization of ABCB1a on apical cell surfaces, and ABCC5a on basolateral surfaces. Conclusions: Embryos use many ABC transporters with predicted functions in cell signaling, lysosomal and mitochondrial homeostasis, potassium channel regulation, pigmentation, and xenobiotic efflux. Detailed characterization of ABCB1a and ABCC5a revealed that they have different temporal and spatial gene expression profiles and protein localization patterns that correlate to their predicted functions in protection and development, respectively. *Developmental Dynamics 241:1111–1124, 2012.* © 2012 Wiley Periodicals, Inc.

Key words: ABC transporter; sea urchin; protection; signaling; cellular defenses; multidrug; embryo; gene expression

Key findings:

- Twenty *ABCB*, *ABCC*, and *ABCG* genes are expressed during the first 58 hr of sea urchin development.
- Expressed ABC transporter genes have predicted functions in cell signaling, lysosomal and mitochondrial homeostasis, potassium channel regulation, pigmentation, and xenobiotic efflux.
- *ABCB1a* mRNA is 10–100 times more abundant than mRNA of other ABC transporters.
- *ABCB1a* is expressed in all cells of the embryo, while *ABCC5a* is expressed only in mesodermal precursors.
- ABCB1a protein localizes to the apical membrane of the embryo, while ABCC5a is expressed on basolateral membranes of polarized cells.

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INTRODUCTION

ATP-binding cassette (ABC) transporters are a conserved family of membrane proteins that use ATP to move compounds across membranes in both adult and embryonic cells (Higgins, 1992; Elbling et al., 1993; Dean et al., 2001; Borst and Elferink, 2002). They transport peptides, metals, xenobiotics, and ions necessary for homeostasis, protection, and signaling. The ABC transporter family comprises eight subfamilies in sea urchins (ABCA to ABCH) and seven

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in mammals (ABCA to ABCG). Much of the functional information about these transporters pertains to the ABCB, ABCC, and ABCG "multidrug efflux" subfamilies and their roles in diseases including cancer, cholestasis, and cystic fibrosis. For example, increased expression of ABCB1 (P-glycoprotein, Pgp), ABCC1 (multidrug resistance protein 1, MRP1), and ABCG2 (breast cancer resistance protein, BCRP) in cancer cells leads to acquired drug resistance (Borst and Elferink, 2002). In contrast, reduced surface expression of the ABC transporters ABCB11 (bile salt export pump, BSEP) and ABCC7 (cystic fibrosis transmembrane conductance regulator, CFTR) leads to cholestasis and cystic fibrosis, respectively (Dean et al., 2001; Borst and Elferink, 2002).

While ABC transporters are well studied in disease, relatively little is known about their functions in embryos. In the *Strongylocentrotus purpuratus* genome, there are >100 ABC transporter gene predictions (Cameron et al., 2009, http://spbase.org). Whole-genome tiling arrays revealed that these genes are extensively expressed in the first 5 days of embryonic development. For example, >80% of *ABCC* genes were detected, a level that is ~30% higher than the overall level of gene usage across the genome (Goldstone et al., 2006; Samanta et al., 2006). This high expression of transporters may be necessary to protect the embryo from xenobiotics. Consistent with this idea, sea urchin embryos possess both ABCB and ABCC transport activities that protect them from vinblastine (Hamdoun et al., 2004) and inorganic mercury (Bosnjak et al., 2009). Similarly, ABCB1 (Pgp) protects mouse embryos from xenobiotics such as doxorubicin and mitomycin C (Elbling et al., 1993).

This high usage of ABC transporters could also indicate that they function in cell specification through efflux of morphogens. For example, *Drosophila melanogaster* mdr49 (an ABCB transporter) protects flies from colchicine toxicity (Wu et al., 1991), and it also transports signaling molecules. *Dm*-mdr49 is expressed in the embryonic mesoderm where it effluxes a chemoattractant that directs germ cell migration to the somatic gonad (Ricardo and Lehmann, 2009). Similarly, human ABCC1 (MRP1) is a xenobiotic transporter, but when expressed heterologously in *Caenorhabditis elegans*, it rescues defects in dauer larva formation induced by removal of the endogenous transporter (Yabe et al., 2005).

The goal of this study was to characterize the gene expression and protein localization of ABC transporters during embryonic development of sea urchins. Of the 40 manually annotated *ABCB*, *ABCC*, and *ABCG* genes, we found 20 to be expressed during the first 3 days of development. We quantified the number of transcripts per egg/embryo for six transporter genes and found that mRNA of *ABCB1a* was 10–100 times more abundant than that of other measured transporters. In situ hybridization of *ABCB1a* and *ABCC5a* revealed that *ABCB1a* was ubiquitously expressed in all cells, while *ABCC5a* was expressed only in a subset of mesodermal precursors. ABCB1a protein primarily localized to the apical membrane of polarized epithelial cells, while ABCC5a was found on basolateral membranes. The spatial gene expression and protein localization patterns of ABCB1a and ABCC5a are consistent with predicted differences in protection versus signaling, respectively. Our results highlight the importance of ABCB, ABCC, and ABCG transporters in a wide range of developmental functions, ranging from protection against xenobiotics to control of cell signaling and differentiation.

RESULTS

ABC Transporter Genes Expressed in Early Development

We measured the dynamics of *ABCB*, *ABCC*, and *ABCG* gene expression during the first three days of sea urchin development, from unfertilized egg through the early prism stage. Embryos expressed 20 transporters including those potentially responsible for xenobiotic efflux, ion movement, and transport of signaling molecules (Table 1). To assess similarity of the detected genes to other well-characterized ABC transporters, we used Blastp to compare their predicted peptide sequences (Spbase.org, genome version 3.1) with other proteins in NCBI. Embryos expressed eight *ABCB* transcripts including three genes related to multidrug pumps, one of which could be related to bile salt export pump (BSEP), four mitochondrial transporters, and one transporter associated with antigen processing (Tap)-like gene. Among the eight *ABCC* genes detected, one was a homolog of a well-known multidrug pump, four genes encoded transporters with both xenobiotic and signaling molecule substrates, and three genes were similar to the potassium channel regulating protein, sulfonylurea receptor-2 (SUR2). Finally, four *ABCG* genes were expressed, including one encoding a xenobiotic pump, one similar to uncharacterized transporters from insects, and two transporters homologous to *Drosophila* White.

Temporal Patterns of ABC Transporter Gene Expression

All of the ABCB, ABCC, and ABCG mRNAs showed little change in abundance from the egg to early blastula stage (Figs. 1, 2, Supp. Table S1, which is available online). The first significant changes in expression (fold change <0.5 or >2) occurred at hatching, consistent with the large synthesis and turnover of mRNA that occurs at this time (Davidson, 1986). For example, transcripts of 13 ABC transporters decreased at hatching, presumably due to turnover of maternally derived mRNA (i.e., synthesized during oogenesis and present in the egg before fertilization).

All 20 ABC transporter genes were generally expressed in one of three temporal patterns: (1) transporter expression is present from egg on, decreases at hatching, then is restored thereafter (Fig. 2, blue lines), (2) transporter transcripts are undetectable in early development and rapidly appear at a distinct developmental time point (Fig. 2, orange lines), and (3) transporter expression is robust from egg on and increases steadily throughout development (Fig. 2, green lines). Thirteen genes showed Group 1 patterns (blue, Fig. 2A–H,J,N–P,R) including *ABCB1a*, *ABCB1b*, *ABCB4a*, *ABCB6*, *ABCB7*, *ABCB8*, *ABCB9a*, *ABCB10a*, *ABCC4a*, *ABCC9a*, *ABCC9b*, *ABCC9d*, and *ABCG9*. After hatching, *ABCB1b*, *ABCB4a*, *ABCB7*, *ABCB10a*, *ABCC9b*,

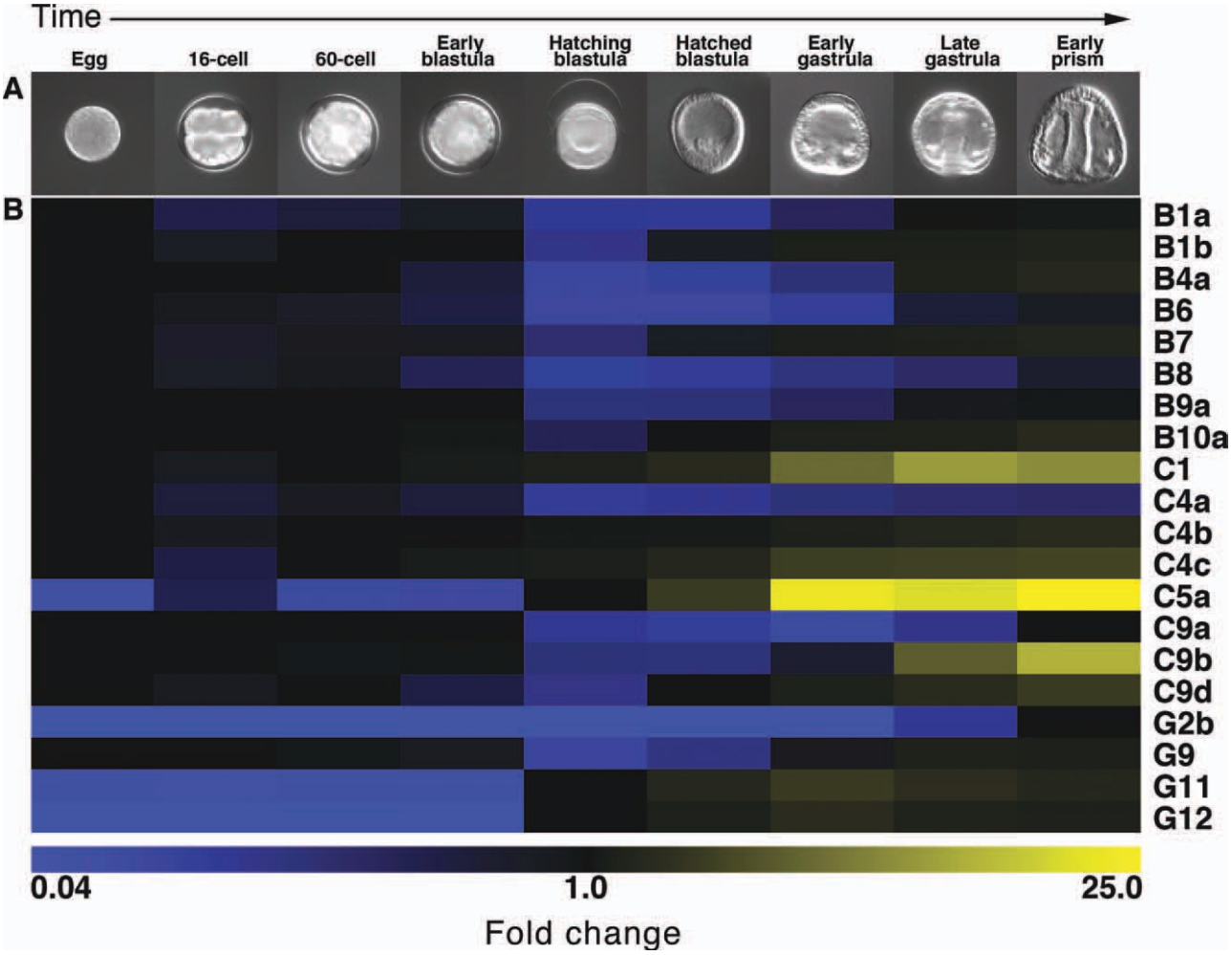

Fig. 1. Developmental stages surveyed and relative ATP-binding cassette (ABC) transporter gene expression. **A**: Differential interference contrast (DIC) micrographs depict the developmental stages included in the gene expression survey. **B**: The heat map shows quantitative real-time polymerase chain reaction (qPCR) data expressed as fold change from the reference stage (i.e., earliest detectable stage). The reference stage is egg in all transporters except *ABCC5a*, *ABCG2b*, *ABCG11*, and *ABCG12*. The reference stage is hatching blastula for *ABCC5a*, *ABCG11*, and *ABCG12*. The reference stage is early prism for *ABCG2b*. All data represent the average of progeny from four females (N = 4).

ABCC9d, and *ABCG9* all increased >2-fold. The second most common expression pattern was the Group 2 pattern (orange, Fig. 2M,Q,S,T) found for *ABCC5a*, *ABCG2b*, *ABCG11*, and *ABCG12*. *ABCC5a*, *ABCG11*, and *ABCG12* were first detected at the hatching blastula stage, while *ABCG2b* was not detected until the early prism stage. Group 3 (green, Fig. 2I,K,L) included three transporters, *ABCC1*, *ABCC4b*, and *ABCC4c*, that increased >2-fold by hatching (*ABCC1*, *ABCC4c*) or early gastrula (*ABCC4b*).

Temporal expression patterns of paralogs.

Many *S. purpuratus* ABC transporters have multiple paralogs. For example, *ABCB1* has 10 paralogs, *ABCC5* has 16, *ABCC9* has 14, and *ABCG2* has five. To gain insight into the potential functions of these paralogs, we examined whether the expression patterns of detected paralogs were temporally synchronous. For *ABCB1a* and *ABCB1b*, expression was fairly uniform with both decreasing at hatching (Fig. 2A,B). In contrast, the *ABCC4* paralogs were asynchronous with *ABCC4a* decreasing at hatching then slowly restoring expression levels, while *ABCC4b* increased >2-fold at early gastrula, and *ABCC4c* increased >2-fold at hatching (Fig. 2J–L). Finally, while the *ABCC9* paralogs all decreased expression at hatching and restored expression levels later in development, only *ABCC9b* and *ABCC9d* increased >2-fold (Fig. 2N–P).

Quantification of ABC Transporter mRNAs

We quantified six ABC transporter transcripts using cDNA standards (Fig. 3; Supp. Table S1) to determine their abundance. These six genes represented each of the three common expression profiles. The genes included *ABCB1a*, *ABCB4a*, and *ABCC9a* from Group 1, *ABCC5a* and *ABCG2b* from Group 2, and *ABCC1* from Group 3. *ABCB1a* was the most abundantly expressed transporter, averaging 12,878 transcripts per egg/embryo and ranging from 6,223 copies per hatched blastula to 20,135 copies per late gastrula embryo (Fig. 3A; Supp. Table S1). *ABCB4a* ranged from 465 copies in hatching blastulae to 4,157 copies

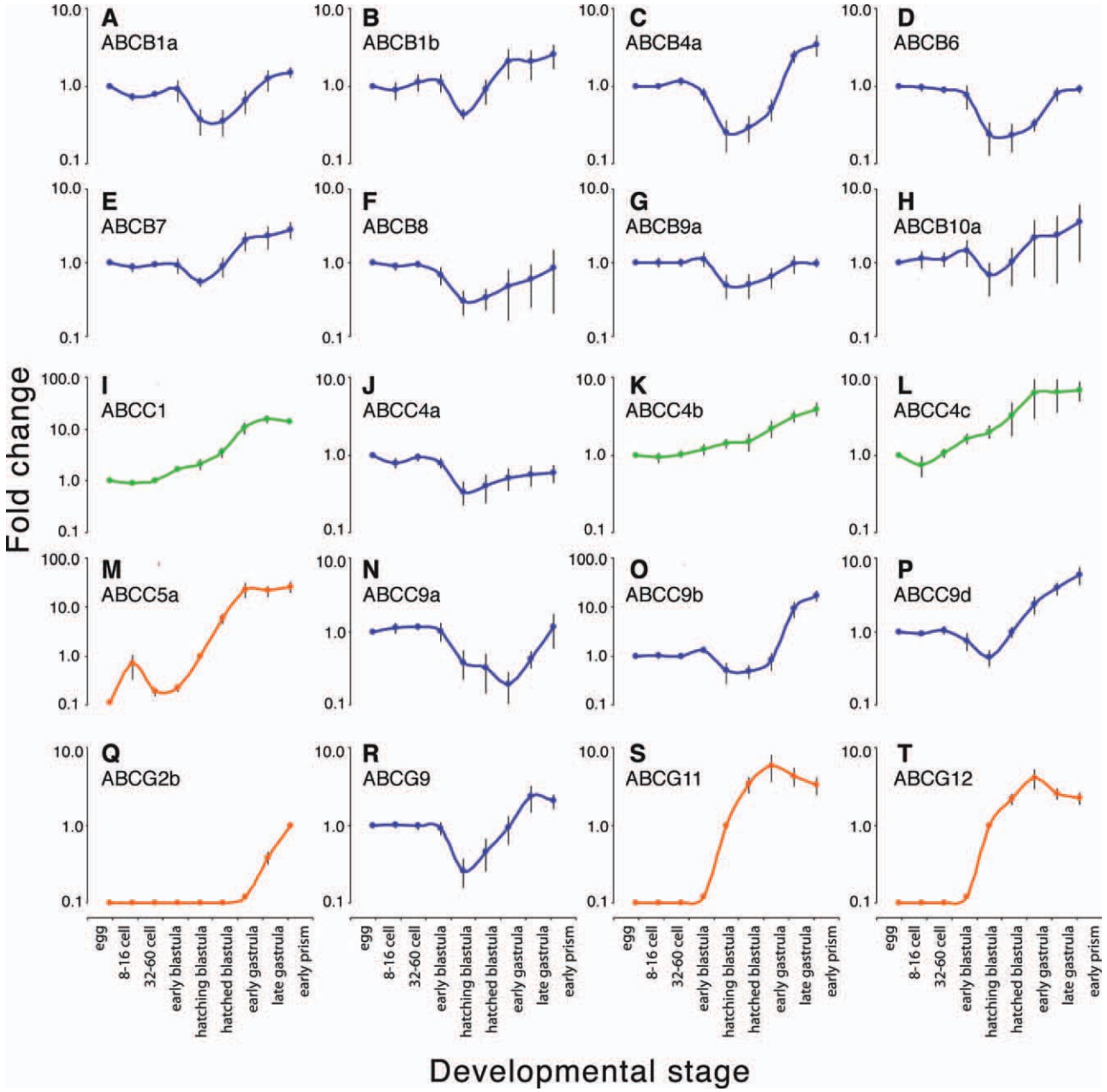

Fig. 2. Relative ABC transporter gene expression during sea urchin development. Data depicted in Figure 1 is presented as individual graphs on a logarithmic scale. Expression profiles are shown in three color-coded groups. Group 1 is shown in blue, Group 2 in orange, and Group 3 in green. N = 4, error bars represent standard error.

per early prism embryo with an average of 1,643 copies (Fig. 3B; Supp. Table S1). *ABCC9a* had an average of 1,532 transcripts per egg/embryo, ranging from 468 copies in early gastrulae to 2,481 copies in 16-cell embryos (Fig. 3E; Supp. Table S1). Both *ABCC5a* and *ABCG2b* could not be detected until they exceeded ~100 transcripts per embryo. *ABCC5a* reached this threshold with 343 copies at hatching, and it peaked at the early prism stage with 7,871 copies per embryo (Fig. 3D; Supp. Table S1). *ABCG2b* transcripts could only be accurately quantified at the latest stage surveyed, when they reached 186 copies per early prism embryo (Fig. 3F; Supp. Table S1). *ABCC1* transcripts were present at 590 copies per egg and 517 copies per 16-cell embryo, then increased through development peaking at 7,471 copies per late gastrula stage embryo (Fig. 3C; Supp. Table S1).

Spatial Patterns of ABC Transporter Gene Expression

Next, we used whole-mount in situ hybridization (WMISH) to characterize spatial expression of five ABC transporter genes. Two genes, *ABCB1a* and *ABCC5a*, showed clear localization patterns. *ABCB4a*, *ABCC1*, and *ABCC9a* were not detected, presumably because their messages were insufficiently abundant and/or widely dispersed.

Temporal expression analyses described above showed that *ABCB1a*, which has predicted protective functions, was the most abundant mRNA (Fig. 3A). In contrast, *ABCC5a* mRNA was undetectable in early development but increased dramatically at a specific developmental stage (Fig. 3D), a pattern that is commonly observed for developmental genes such as *Nodal*, *HesC*, and *Delta* (Nam et al., 2007; Revilla-i-Domingo et al., 2007). Consistent with a predicted function in protection against toxicants, WMISH showed *ABCB1a* was ubiquitously expressed in all cells throughout development (Fig. 4E–H). At the gastrula stage, *ABCB1a* was detected with such intense staining on the ectoderm and endoderm that it was difficult to determine if there was uniform mesodermal expression (Fig. 4H).

In contrast, *ABCC5a* transcripts were undetectable early in development (Fig. 4I), then were expressed only in a subset of embryonic cells after hatching (Fig. 4J–L). *ABCC5a* was expressed in mesodermal cells (Fig. 4K,L) in a pattern consistent with the veg2 lineage and their descendants, the (nonskeletogenic) secondary mesenchyme cells (SMCs; Peter and Davidson, 2009a). In hatched mesenchyme blastulae, *ABCC5a* mRNA was detected in the vegetal pole of the embryo and was absent from primary mesenchyme cells (Fig. 4J). During gastrulation, *ABCC5a*-expressing cells dispersed and were ultimately incorporated into the ectoderm in a pattern similar to that of pigment cells (Fig. 4K,L; Ransick et al., 2002; Peter and Davidson, 2009a). In addition, the temporal expression pattern of *ABCC5a* matches those of *ABCG11* and *ABCG12*, homologs of the transporter necessary for eye pigmentation in *Drosophila*, *Dm*-White (Ewart et al., 1994; Mackenzie et al., 2000). Thus, ABCC5a could be involved in the formation or function of pigment cells in sea urchin embryos.

Cellular Localization of ABCB1a and ABCC5a Proteins

We expressed fluorescent-protein fusions of ABCB1a and ABCC5a in the sea urchin embryo to determine their cellular localization (Fig. 5). ABCB1a-mCitrine protein localized to the apical membrane of the ectoderm in hatched mesenchyme blastulae (Fig. 5Aii). This transporter coated the outside surface of the embryo and was seen on the surfaces of apical microvilli (Fig. 5Bii). In contrast, ABCC5a-mCherry protein was absent from the apical cell surface and was instead localized on the basolateral cell membranes (Fig. 5Aiii, Biii). Almost no colocalization of ABCB1a-mCitrine and ABCC5a-mCherry proteins was observed in polarized epithelial cells (Fig. 5Biv), except for some slight overlap at the vegetal pole of the embryo (Fig. 5Aiv).

Our findings from both WMISH and FP-overexpressions indicate that at the hatched blastula stage, endogenous *ABCB1a* transcripts are present in all embryonic cells (Figs. 4F, 6A), while ABCB1a-mCitrine protein localizes on the apical membrane of all ectodermal cells (Fig. 5Aii). This indicates that in blastulae, endogenous ABCB1a protein is also present on the apical side of all ectodermal cells (Fig. 6B, green). This places ABCB1a proteins in direct contact with the environment, where they can directly efflux unwanted chemicals from the embryo.

In contrast, at the same developmental stage, *ABCC5a* transcripts are detected exclusively in a subset of vegetal cells (Figs. 4J, 6A) likely to be part of the veg2 lineage (Peter and Davidson, 2009a). ABCC5a-mCherry protein is not detected at the apical surface of the embryo but is instead localized on basolateral membranes of polarized cells, which are not in direct contact with the environment (Fig. 5Aiii). Thus, we propose that endogenous ABCC5a protein is expressed on basolateral membranes of vegetal cells in hatched blastulae (Fig. 6B, red). This position in the embryo suggests that ABCC5a is not involved in protective efflux of environmental chemicals.

DISCUSSION

Our results demonstrate that sea urchin embryos use many ABC transporters in early development. In the first three days of development, 20 ABC transporters were expressed including those with predicted functions in cell signaling, mitochondrial and lysosomal homeostasis, potassium channel regulation, pigmentation, and xenobiotic efflux (Table 1). In situ hybridization and fluorescent protein fusion expression of ABCB1a and ABCC5a revealed significant differences in location and timing of expression of these two transporters that correlate with predicted differences in their functions.

Xenobiotic transport.

Sea urchin embryos expressed homologs of all three major types of multidrug transporters including ABCB1, ABCC1, and ABCG2. Among the detected ABCB transporters, *Sp-ABCB1a*, *Sp-ABCB1b*, and *Sp-ABCB4a* are similar to the human multidrug resistance transporter, P-glycoprotein (Pgp). This transporter protects cells by effluxing a wide range of mildly hydrophobic molecules (Dean et al., 2001; Fletcher et al., 2010), and its substrate poly-specificity is mediated by a large binding pocket with multiple drug binding sites (Aller et al., 2009). ABCB1/Pgp-mediated xenobiotic efflux activity is essential for protecting hematopoietic stem cells (Smeets et al., 1997) and various mammalian (Elbling et al., 1993) and invertebrate embryos including sea urchins (Toomey and Epel, 1993; Hamdoun et al., 2004).

Previous studies indicated that sea urchins have high levels of ABCC-like multidrug efflux activity (Hamdoun et al., 2004). We found that embryos expressed *Sp-ABCC1*, a homolog of multidrug resistance protein 1 (MRP1), which could mediate this activity. In addition to direct efflux of toxic molecules, ABCC1/MRP1 effluxes glutathione-conjugates and, therefore, can also transport hydrophilic toxicants such as metals (Leslie et al., 2005; Fletcher et al., 2010; Chen and Tiwari, 2011; He et al., 2011). ABCC1 can also maintain cellular redox homeostasis by means of GSH and GSSG transport (Leslie et al., 2001), and it regulates inflammation and dendritic cell migration by means of transport of leukotriene

TABLE 1. ABC Transporter Genes Detected in Sea Urchin Development and Functions of Their Homologs[a]

Gene	Gene ID	Annotated peptide length (aa)	Homologs	Blastp score	Also known as	Cellular membrane localization	Function (substrates)	References
Sp-ABCB1a	SPU_001752	1329	*H. sapiens* ABCB1	0.0	CLCS; MDR1; P-GP; PGY1	Apical	Xenobiotic efflux (anthracyclines, vinca alkaloids, taxanes, epipodophyllotoxins, mitoxantrone)	(Dean et al., 2001; Leslie et al., 2005; Fletcher et al., 2010)
Sp-ABCB1b	SPU_002431	1079	*H. sapiens* ABCB11	0.0	BSEP; PGY4; SPGP	Apical	Bile salt transport (Taurocholate, Glycocholate, Taurochenodeoxycholate)	(Stieger et al., 2007)
Sp-ABCB4a	SPU_007014	1235	*H. sapiens* ABCB1	0.0	CLCS; MDR1; P-GP; PGY1	Apical	Xenobiotic efflux (anthracyclines, vinca alkaloids, taxanes, epipodophyllotoxins, mitoxantrone)	(Dean et al., 2001; Leslie et al., 2005; Fletcher et al., 2010)
			H. sapiens ABCB4	0.0	GBD1; MDR2; MDR3; PGY3	Apical	Phospholipid flippase (phosphatidylcholine); Bile salt transport; Xenobiotic efflux (Anthracyclines, vinca alkaloids, taxanes, epipodophyllotoxins, mitoxantrone)	(Dean et al., 2001; Borst and Elferink, 2002; Fletcher et al., 2010)
Sp-ABCB6	SPU_018342	776	*H. sapiens* ABCB6	0.0	ABC; PRP; MTABC3	Outer mitochondrial	Iron transport	(Dean et al., 2001; Zutz et al., 2009)
Sp-ABCB7	SPU_003241	651	*H. sapiens* ABCB7	0.0	ABC7; ASAT; Atm1p	Inner mitochondrial	Cytosolic Fe/S transport; Iron homeostasis	(Dean et al., 2001; Zutz et al., 2009)
Sp-ABCB8	SPU_024666	469	*H. sapiens* ABCB8	4e-97	MABC1; M-ABC1	Inner mitochondrial	Oxidative stress protection	(Zutz et al., 2009)
Sp-ABCB9a	SPU_026825	398	*H. sapiens* ABCB9	8e-160	TAPL	Lysosomal	Peptide transport	(Zhao et al., 2006; Bangert et al., 2011)
Sp-ABCB10a	SPU_016850	623	*H. sapiens* ABCB10	0.0	M-ABC2; MTABC2	Inner mitochondrial	Iron transport; possible peptide transport & antigen processing	(Herget and Tampé, 2007; Chen et al., 2009; Zutz et al., 2009)
Sp-ABCC1	SPU_026395	1025	*H. sapiens* ABCC1	0.0	MRP; ABCC; GS-X; MRP1	Basolateral	Xenobiotic efflux (GSH-conjugates, anthracyclines, mitoxantrone, vinca alkaloids, imatinib, epopodophyllotoxins, camptothecins, colchicines, metals, methotrexate, Etoposide-glucuronide, Doxorubicin-GS, glutathione disulfide (GSSG)); Signaling & homeostasis (GSH-conjugates, leukotrienes, prostaglandins, sphingosine-1-phosphate, bilirubin, estradiol 17β-D-glucuronide)	(Leslie et al., 2005; Fletcher et al., 2010; Chen and Tiwari, 2011; He et al., 2011)

TABLE 1. (Continued)

Gene	Gene ID	Annotated peptide length (aa)	Homologs	Blastp score	Also known as	Cellular membrane localization	Function (substrates)	References
Sp-ABCC4a	SPU_020669	1411	*H. sapiens* ABCC4	0.0	MRP4; MOATB	Apical or Basolateral (tissue dependent)	Xenobiotic efflux (nucleosides, thiopurines, PMEA, methotrexate, anti-HIV nucleoside analogues, camptothecins); Signaling & homeostasis (leukotrienes, prostaglandins, thromboxane, cyclic nucleotides)	(Fletcher et al., 2010; Chen and Tiwari, 2011)
Sp-ABCC4b	SPU_024191	1214	*H. sapiens* ABCC4	1e-103				
Sp-ABCC4c	SPU_002411	1174	*H. sapiens* ABCC4	0.0				
Sp-ABCC5a	SPU_023723	1424	*H. sapiens* ABCC5	0.0	MRP5; SMRP; MOATC	Basolateral	Organic anion transport (acidic organic dyes, dinitrophenylglutathione); Xenobiotic efflux (methotrexate, cisplatin, PMEA, AZT, daunorubicin, doxorubicin, gemcitabine); Signaling & homeostasis (cyclic nucleotides)	(Borst and Elferink, 2002; Fletcher et al., 2010; Chen and Tiwari, 2011)
Sp-ABCC9a	SPU_025903	1585	*H. sapiens* ABCC9	0.0	SUR2		Potassium channel regulation	(Bryan et al., 2007)
Sp-ABCC9b	SPU_028797	1497	*H. sapiens* ABCC9	0.0				
Sp-ABCC9d	SPU_007764	1481	*H. sapiens* ABCC9	0.0				
Sp-ABCG2b	SPU_014013	448	*H. sapiens* ABCG2	7e-138	MRX; MXR; ABCP; BCRP; BMDP	Apical	Xenobiotic efflux (mitoxantrone, camptothecins, anthracyclins, bisantrene, imatinib, methotrexate, flavopiridol, epipodophyllotoxins); Stem cell protection & maintenance	(Leslie et al., 2005; Krishnamurthy and Schuetz, 2006; Fletcher et al., 2010)
Sp-ABCG9	SPU_012874	485	*D. melanogaster* E23	1e-81	Early gene at 23		Ecdysone signaling, circadian rhythm	(Itoh et al., 2011)
Sp-ABCG11	SPU_020849	590	*D. melanogaster* White	5e-109	white; DMWHITE; EG:BACN33B1.1	Pigment granular	Eye color determinant (pigment metabolites)	(Ewart et al., 1994; Mackenzie et al., 2000)
Sp-ABCG12	SPU_015080	677	*D. melanogaster* White	0.0				

[a]Blastp score indicates E value.

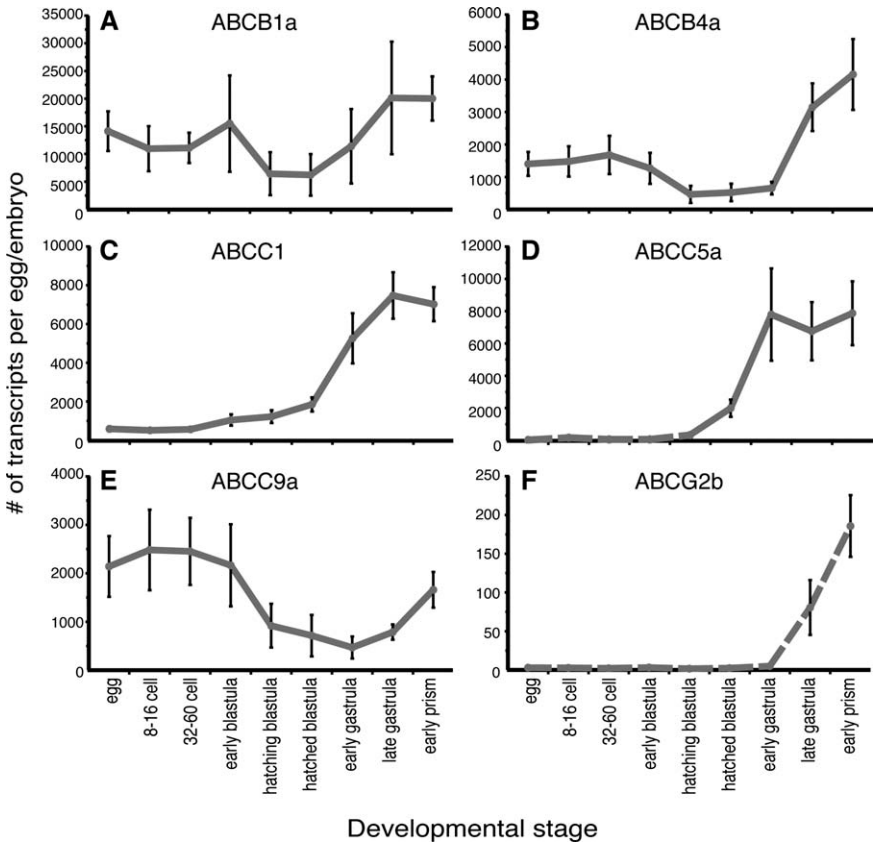

Fig. 3. Number of ABC transporter transcripts per egg/embryo. The number of mRNA copies per egg/embryo is shown. Transcript copies were determined using a standard curve to quantify the reference point (egg for *ABCB1a*, *ABCB4a*, *ABCC1*, and *ABCC9a*; hatching blastula for *ABCC5a*; early prism for *ABCG2b*), and by applying fold change values (Fig. 1, Fig. 2, Supp. Table S1) to quantify all other stages. Dashed lines (D, F) indicate where transcripts were below the threshold of detection using qPCR (i.e., <100 copies per egg/embryo). N = 4, error bars represent standard error.

LTC_4 (Leslie et al., 2005). Because ABCC1 performs both protective and signaling functions in mammals, it could also be a dual-functioning transporter in sea urchin development.

Finally, sea urchin embryos expressed *Sp-ABCG2b*, a homolog of human breast cancer resistance protein (BCRP). ABCG2/BCRP is a xenobiotic transporter known to mediate drug resistance by effluxing anticancer drugs such as mitoxantrone (Leslie et al., 2005; Krishnamurthy and Schuetz, 2006). In addition to protection, ABCG2 may also maintain multipotency of hematopoietic stem cells (Zhou et al., 2001; Bunting, 2002). Consistent with this hypothesis, ABCG2 is involved in porphyrin homeostasis and contributes to self-renewal of mouse embryonic stem cells (Susanto et al., 2008).

Mitochondrial transport.

An interesting finding of our study was the expression of four homologs of mitochondrial transporters, *Sp-ABCB6*, *Sp-ABCB7*, *Sp-ABCB8*, and *Sp-ABCB10a*. In sea urchin embryos, mitochondria are important for both energetics and oral–aboral specification (Coffman et al., 2009). The genes expressed are homologous to mammalian mitochondrial half-transporters likely to function in Fe/S cluster transport, iron homeostasis, heme biosynthesis, peptide transport, and oxidative stress protection (Herget and Tampé, 2007; Zutz et al., 2009; Chen et al., 2009). In mammals, three of these transporters (ABCB7, ABCB8, and ABCB10) are expressed on the inner mitochondrial membrane, while ABCB6 is thought to localize to the outer mitochondrial membrane (Zutz et al., 2009).

Lysosomal transport.

The final ABCB gene expressed was *Sp-ABCB9a*, which is homologous to the lysosomal Tap-like (TAPL) protein, a transporter of peptides from cytosol into the lysosome. ABCB9/TAPL may perform a homeostatic role such as disposing of accumulated cytosolic peptides (Zhao et al., 2006; Bangert et al., 2011), and it is possibly also involved in antigen processing (Bangert et al., 2011).

Multifunctional transport.

Several of the expressed transporters could have dual functions in the embryo, including the aforementioned example of *Sp*-ABCC1. We detected other predicted multifunctional transporters including *Sp-ABCC4a*, *Sp-ABCC4b*, *Sp-ABCC4c*, and *Sp-ABCC5a*, which are homologs of mammalian transporters with broad specificity for both signaling molecules (e.g., cGMP) and xenobiotics. Like ABCC1, ABCC4/MRP4 transports leukotrienes and is necessary for human dendritic cell migration (Fletcher et al., 2010).

One of the central transporters in this study was *Sp*-ABCC5a, whose homologs remain relatively poorly characterized in any organism. In humans, ABCC5/MRP5 localizes to basolateral membranes of polarized cells, and its mRNA is ubiquitously expressed in adult tissues, though it is highest in skeletal muscle, heart, and brain (Chen and Tiwari, 2011). It has been suggested to be a cGMP transporter (Jedlitschky et al., 2000), although its affinity for this substrate is relatively low (de Wolf et al., 2007). Because *Hs*-ABCC5 may export cyclic nucleotides, one possibility is that *Sp*-ABCC5a plays some role in signaling necessary for morphogenesis or migration of the mesenchyme cells. Alternatively, it may function in xenobiotic efflux, though in other systems it has yet to be demonstrated as toxicologically important (Leslie et al., 2001; Chen and Tiwari, 2011). One possibility is that *Sp*-ABCC5a may be involved in protection against some endogenously produced toxic metabolite.

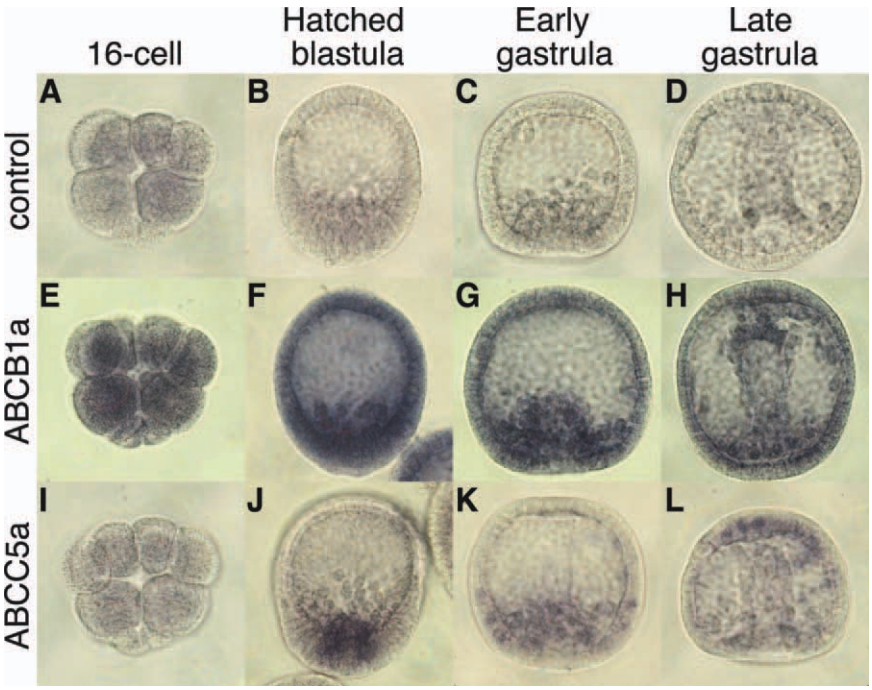

Fig. 4. Spatial expression of ABC transporter genes. Whole-mount in situ hybridization depicts endogenous expression of *ABCB1a* and *ABCC5a*. **A–D**: For controls, digoxigenin (DIG) -labeled sense probe was used. **E–H**: *ABCB1a* is expressed in all cells of embryos. **I–L**: *ABCC5a* mRNA is not detected before hatching (I), after which it is detected in the vegetal pole of hatched mesenchyme blastulae (J), and in mesodermal cells (K,L) in later embryos. A,E,I: 16-cell embryos were fixed at 7 hours post-fertilization (hpf; B,F,J) hatched mesenchyme blastulae at 36 hpf, (C,G,K) early gastrulae at 42 hpf, and (D,H,L) late gastrulae at 48 hpf.

Potassium channel conductance regulators.

Three of the expressed transporter genes, *Sp-ABCC9a*, *Sp-ABCC9b*, and *Sp-ABCC9d*, are homologous to sulfonyl urea receptor 2 (SUR2), which associates with inwardly rectifying potassium channels to regulate insulin secretion in humans (Bryan et al., 2007). Little is presently known about the functions of these channels in development. In *Drosophila*, ABCC9/SUR2 is expressed in trachea and dorsal vessels and is potentially involved in cell migration (Nasonkin et al., 1999).

White transporter homologs.

Finally among the genes expressed, *Sp-ABCG11* and *Sp-ABCG12* are homologs of the White half-transporter, which transports precursors or metabolic intermediates of pigment to control eye color in *D. melanogaster* (Ewart et al., 1994; Mackenzie et al., 2000). Like the ABCB half-transporters, White protein is not localized on the cell membrane, but instead is

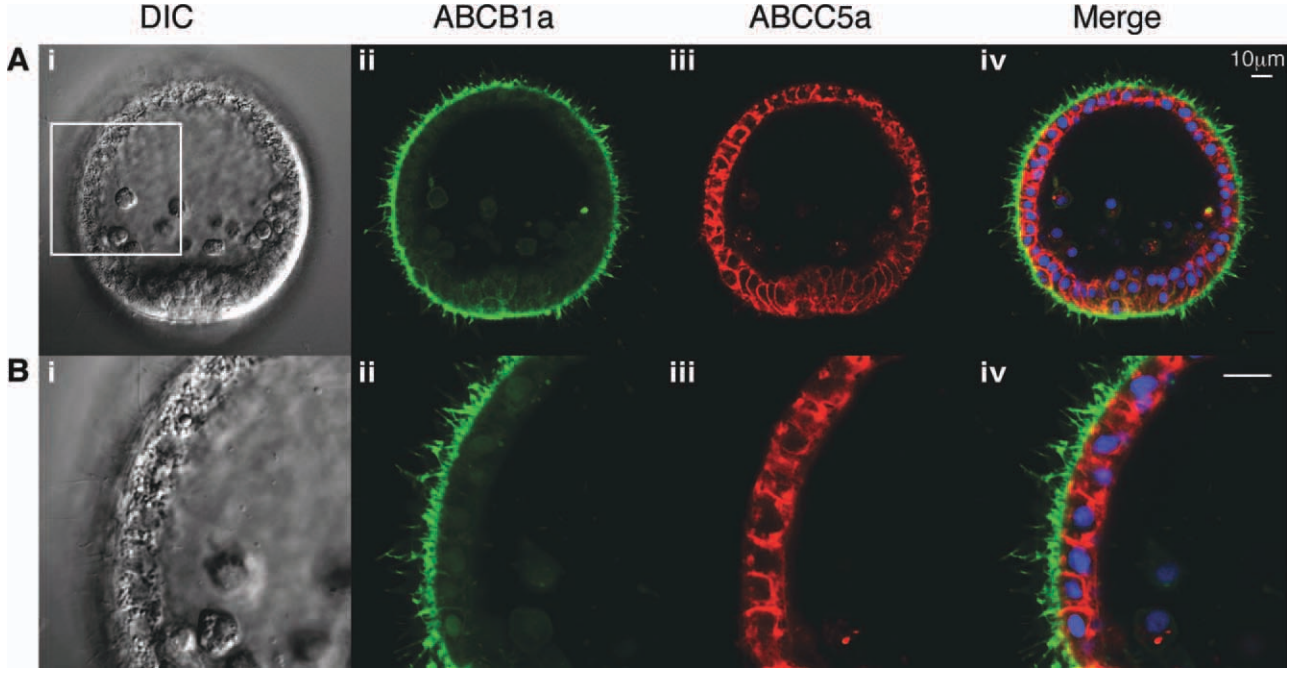

Fig. 5. Localization of ABCB1a and ABCC5a proteins. Micrographs show exogenous ABCB1a and ABCC5a localization from expression of fluorescent-protein fusions. Representative embryos are shown. **B** is an inset from the embryo shown in **A**. mRNA from ABCB1a-mCitrine (ii, green) and ABCC5a-mCherry (iii, red) were injected into fertilized eggs, then embryos were grown to mesenchyme blastulae for imaging. iv: Merged channel includes Histone H2B-CFP (blue), injected as a nuclear marker. ABCB1a-mCitrine protein localizes to the apical surface of the embryo, while ABCC5a-mCherry localizes on the basolateral cell surfaces.

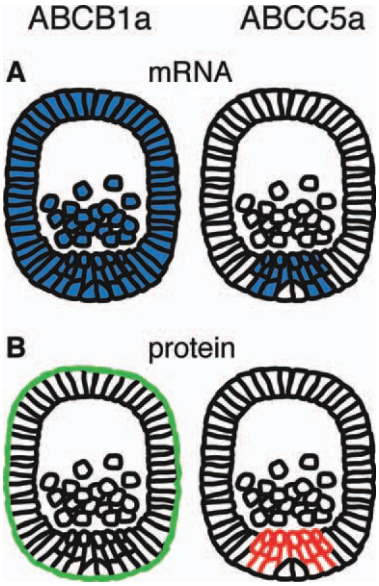

Fig. 6. Model of endogenous ABCB1a and ABCC5a protein localization. **A**: A cartoon represents whole-mount in situ hybridization (WMISH) transcript localization shown in Figure 4F,J. **B**: By combining this with FP-fusion ABCB1a and ABCC5a protein localization data shown in Figure 5A, we propose a model for endogenous ABCB1a (green) and ABCC5a (red) expression. ABCB1a is primarily expressed on the apical membrane of polarized epithelial cells, while ABCC5a is expressed on the basolateral membranes of secondary (nonskeletogenic) mesenchyme cells.

found on intracellular vesicles (Mackenzie et al., 2000; Evans et al., 2008) where it may also transport cGMP (Evans et al., 2008). In *Drosophila* embryos, *white* transcripts are detected in the Malphigian tubules coincident with the onset of cell differentiation (Fjose et al., 1984).

Transporters with unresolved classification.

We found two genes that may have different homologs than those indicated by their original names. For example, although the name *Sp-ABCB1b* indicates similarity to human ABCB1/Pgp, the top NCBI Blastp hits were chicken ABCB1 (CMDR1; e = 0.0) and human ABCB11 (bile salt export pump, BSEP; e = 0.0). ABCB11 transports bile salts across the canalicular plasma membrane (Stieger et al., 2007). Chicken *Mdr1* is expressed in the thymus and bursa of embryos and may participate in lymphoid differentiation of T and B cells (Petrini et al., 1995). Given that sea urchins lack a direct ABCB11 homolog, it is possible that *Sp*-ABCB1b transports sterols similar to bile salts.

Similarly, although *Sp-ABCC9b* is homologous to ABCC9/SUR2 (e = 0.0), Blastp indicates it is equally similar to ABCC2/multidrug resistance protein 2 (ABCC2; e = 0.0). ABCC2 is related to ABCC1, and as such it effluxes both signaling molecules and xenobiotics (Leslie et al., 2001; Fletcher et al., 2010). Thus, one possibility is that *Sp*-ABCC9b has different functions than those predicted by its designation as an ABCC9/SUR.

Differential Regulation of Transporters

Given this great diversity of ABC transporters present in embryos, it seems plausible that multiple modes of regulation are used to maintain and modulate their membrane activity through development. For example, our results with ABCB1a and ABCC5a indicate that they are likely to be under different modes of regulation.

ABCB1a transcripts are abundant throughout development (Fig. 3A; Supp. Table S1) and are strongly detected in all cells of embryos including the primary mesenchyme (Fig. 4F). Yet, while ABCB1a-mCitrine accumulates to high levels on surfaces of ectodermal cells, it is expressed weakly on surfaces of primary mesenchymal cells (Fig. 5Aii, Bii). This could indicate that ABCB1a is post-transcriptionally regulated and that levels of its mRNA do not necessarily correlate to surface levels of the protein. Additional evidence for post-transcriptional regulation of ABCB1a comes from the observation that efflux activity increases after fertilization of sea urchin eggs, even with exposure to inhibitors of transcription and translation (Hamdoun et al., 2004). This indicates that in very early development, efflux activity is post-translationally controlled, and it is possible that ABCB1a is similarly regulated throughout development. Alternatively, primary mesenchyme cells may have less ABCB1a-mCitrine due to membrane turnover associated with the epithelial to mesenchymal transition (EMT) (Wu et al., 2007).

In contrast, *ABCC5a* expression is tightly temporally and spatially controlled (Figs. 3D, 4J), expressed only after hatching and exclusively in a subset of veg2 cells and their descendants. This suggests that *ABCC5a* is transcriptionally regulated. Consistent with this hypothesis, in MCF7 cells, *Hs-ABCC5* expression is regulated by the EMT-inducing transcription factors Snail, Twist and FOXC2 (Saxena et al., 2011). These are important developmental transcription factors that could interact with *Sp-ABCC5a*. Our future studies will address this possibility and probe the role of *Sp*-ABCC5a in protection, specification, and/or functions of mesodermal cells.

Conclusions

Collectively, our results highlight the diversity of ABC transporters necessary for sea urchin development and provide a foundation for exploring their biology. The characterization of ABCB1a and ABCC5a emphasizes differences in spatial and temporal expression of ABC transporters, and the relation of these differences to predicted functions. Clearly, ABC transporters are more than protective transporters in embryogenesis, and exquisite regulation of membrane function by expression of transporters is likely central to homeostasis, protection, and signaling during development. Our future work will focus on major developmental transitions to address the regulation and function of ABCC5a in protection and/or specification of embryonic cells.

EXPERIMENTAL PROCEDURES

Animals

Purple sea urchins, *Strongylocentrotus purpuratus*, were collected off the coast of San Diego, California, kept in 12°C running seawater, and fed *Macrocystis pyrifera*. Gametes were collected according to standard procedures (Foltz et al., 2004). Eggs were collected in raw seawater, passed through a 120-μm nitex filter, and washed in filtered seawater (FSW). For RNA isolation and in situ analyses, a 500 ml solution of 1% packed eggs in FSW was fertilized with 5 μl

sperm (in FSW). Fertilization was visually confirmed, and only batches with >90% fertilization were used for experiments. Embryos were washed twice in FSW to remove excess sperm, and the culture was grown at a concentration of 500 embryos/ml FSW at 12°C.

RNA Isolation

For all gene expression experiments, total RNA was isolated at nine developmental stages (approximate hours post fertilization, hpf): (1) Unfertilized egg, (2) 16-cell (~6 hpf), (3) 60-cell (~8.5 hpf), (4) early blastula (12–13 hpf), (5) hatching blastula (21–23 hpf), (6) hatched blastula (27–29 hpf), (7) early gastrula (33–35 hpf), (8) late gastrula (50–52 hpf), (9) early prism (55–58 hpf). Experiments were replicated four times with progeny of four females.

Aliquots of cultures were hand-centrifuged to pellet eggs/embryos, and RNA was isolated using a Nucleospin RNA II isolation kit (Macherey-Nagel, Bethlehem, PA) according to the manufacturer's protocol. Yields varied (depending on the density of the pellet) from 3–56 μg total RNA. RNA concentration and purity was determined by spectrophotometry and agarose gel electrophoresis. Only samples with absorbance ratios of ~2.0 (260/280) and ~2.0–2.2 (260/230) with clear major ribosomal subunit bands on gel visualization were used for experiments.

cDNA Synthesis

Reverse transcription was performed using 500 ng of total RNA, 1.5 μM random primer (New England Biolabs, Ipswich, MA), 0.5 mM dNTPs (Fermentas, Glen Burnie, MD), M-MuLV Reverse Transcriptase (New England Biolabs), and RNasin (Promega, Madison, WI) at a final volume of 20 μl.

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qPCR) was performed on a Stratagene MX3000p thermal cycler (Agilent, Santa Clara, CA) with EVA QPCR SuperMix Kit (Biochain, Hayward, CA) according to the manufacturer's protocol. All reactions were run in duplicate. To each 20 μl of cDNA synthesis reaction, 200 μl of nuclease-free water was added for analysis with qPCR. A volume of 3.5 μl was used as template for qPCR with 625 nM of each (forward and reverse) primer in a total reaction volume of 20 μl per well.

Primer Design and Testing

We designed a total of 76 primer pairs: 24 in the ABCB family, 38 in the ABCC family, and 14 in the ABCG family. For each ABC transporter, primers were designed to avoid conserved regions (i.e., nucleotide binding domains). Two primer pairs (forward and reverse) were designed for each transporter gene, and the best pair was selected for each gene. Selected primer pairs are listed in Supp. Table S2. Primers for control genes *Nodal*, *Nanos2*, and *Spz12* were generically designed from existing cDNA annotations. The *Ubiquitin* control primer pair was taken from http://sugp.caltech.edu/SUGP/resources/methods/q-pcr.php.

Primers were tested by amplification with serial dilutions of stock cDNA using the following criteria: confirmation of a "steep" amplification curve, single peak dissociation curve, and correct length (~100 bp) of a single amplicon on an agarose gel (Schmittgen and Livak, 2008). Serial dilutions of cDNA were made in 1×, 4×, 64×, 256×, and 1,024× dilutions with water. Each primer was tested with these dilutions from eggs, 24 hpf, and/or 55 hpf embryos to confirm that threshold cycle (Ct) increased 2 units for each dilution. Primer pairs that did not meet the above criteria were rejected from the study.

Confirmation of Amplicon Specificity to Targeted Genes

Amplicon specificity was confirmed for a subset of primers (*ABCB1a*, *ABCB4a*, *ABCC1*, *ABCC5a*, *ABCC9a*, and *ABCG2b*) by cloning and sequencing the qPCR products from embryos of two females. qPCR amplicons were purified and cloned into a pCR4-TOPO vector (Invitrogen, Life Technologies, Grand Island, NY) according to the manufacturer's protocol, then sequenced (Retrogen, San Diego, CA). The resulting sequences were searched using the Blastn algorithm on SpBase.org in the Sp genome v3.1 database. All inputs mapped exclusively to the targeted gene, confirming the qPCR primers were specific to individual targeted genes.

qPCR Analyses

Gene expression changes are reported as fold differences with respect to the unfertilized egg. For *ABCC5a*, *ABCG2b*, *ABCG11*, and *ABCG12*, egg transcript levels were too low to quantify so expression is reported with respect to the earliest developmental stage at which quantification was possible (hatching blastula for *ABCC5a*, *ABCG11*, and *ABCG12*, and early prism for *ABCG2b*). The formula 2^x was used, where x is the threshold cycle (Ct) number difference between the reference stage (egg, hatching blastula or early prism) and the other stages of development (ΔΔCt method). For example, the prenormalized fold change for *ABCB1a* gene expression at the late gastrula stage is:

$$2^{x}_{\mathrm{ABCB1a}} = 2\hat{}(\mathrm{Ct}^{\mathrm{egg}} - \mathrm{Ct}_{\mathrm{late\ gastrula}})^{\mathrm{ABCB1a}}$$

Results were normalized to *Ubiquitin* according to (Nemer et al., 1991; Juliano et al., 2006; Peter and Davidson, 2009b) such that the reported value reflects the formula:

$$\text{Fold change} = 2^{x}_{\mathrm{ABCB1a}} - 2^{x}_{\mathrm{ubiquitin}}$$

Reported data are an average of four females. Fold changes are significant if they are ±2-fold change from the reference point (0.5 < not significant < 2) (Peter and Davidson, 2009b).

Transcript Copy Number Calculations

Transcript copy numbers per egg/embryo were calculated by quantifying the reference time point against a standard curve generated with dilutions of the sequenced TOPO-cloned qPCR amplicons. We calculated that each qPCR well contained the equivalent of 1.27 eggs/embryos per well based on the following: each *S.*

purpuratus egg/embryo contains between 3.3 ng (Brandhorst, 2004) and 3.0 ± 0.2 ng of total RNA through 60 hpf (Nemer et al., 1984). Using an average of these values (3.15 ng per egg/embryo) and the assumption that 50% of material is lost in converting mRNA to cDNA (Ransick, 2004), we converted 0.5 μg total RNA to cDNA to achieve ~80 embryos worth of cDNA in a 220 μl volume. We used 3.5 μl per qPCR well, corresponding to 1.27 eggs/embryos per well.

Using a DNA molecular weight calculator (www.bioinformatics.org), we determined the molecular weight of each TOPO-cloned qPCR amplicon. We made 4× serial dilutions of these plasmids in water at concentrations equivalent to 1–1,048,576 copies per egg/embryo. We repeated qPCR with the serial dilution series and the reference time point cDNA samples. From the dilution series, we generated a standard curve by applying a nonlinear regression trendline fit (Microsoft Excel) in the format: $Y = ae^{-bX}$ where Y is copies per egg/embryo and X is Ct. Transcript numbers were calculated from these equations.

ABC Transporter Protein Expression

Sp-ABCB1a and *Sp-ABCC5a* cDNAs were cloned from egg and gastrula stage RNA, respectively, by rapid amplification of cDNA ends (RACE; Clontech, Mountain View, CA) according to the manufacturer's protocol. Phusion High-Fidelity DNA polymerase (New England BioLabs) was used for all PCR reactions. Fluorescent proteins mCitrine and mCherry were subcloned into a modified pCS2 vector, and fusions were generated by inserting transporter cDNAs using *Xho*I for an N-terminal FP-ABCB1a fusion (ABCB1a-mCitrine), and *Spe*I for a C-terminal FP-ABCC5a fusion (ABCC5a-mCherry). All constructs were sequenced after cloning (Retrogen). Capped mRNA was made using the mMessage mMachine SP6 kit (Ambion, Life Technologies, Grand Island, NY) according to the manufacturer's protocol. mRNA was injected into fertilized eggs at 2–5% egg volume in a final concentration of 1 mg/ml in water (Lepage and Gache, 2004). Fluorescent protein localization was visualized on a Zeiss LSM-700 laser scanning confocal microscope using a Zeiss Plan APOChromat ×20 air objective (numerical aperture, 0.8; Zeiss, Thornwood, NY). All images were captured using the Zen software suite (Zeiss, revision 5.5) and processed with ImageJ freeware (NIH, Bethesda, MD).

Whole-Mount In Situ Hybridization (WMISH)

Templates for in situ probes were generated by cloning ~1.5 kb of *ABCB1a* or *ABCC5a* into dual promoter pCRII-Topo TA vector (Invitrogen) according to the manufacturer's protocol. PCR was carried out with Phusion High-Fidelity DNA polymerase (New England BioLabs) using ABCB1a-mCitrine and ABCC5a-mCherry as templates. Primers and probe positions are listed in Supp. Table S2. Probe templates were sequenced after cloning (Retrogen).

WMISH was performed following a modified protocol (Ransick, 2004). Briefly, hatched blastulae and later stage embryos were cultured as described. Cleavage-stage embryos were fertilized in 1 mM para-aminobenzoic acid (PABA) and passed through a 60 μm filter to remove the fertilization envelope, then cultured as described. Swimming embryos were pelleted by cooling and gentle centrifugation. A dense aliquot of eggs or embryos was distributed among wells of plastic round-bottom plates to form a monolayer at the base of each well. Specimens were fixed on ice in two steps: (1) 20 min with 0.625% glutaraldehyde in fixation buffer (FB: 32.5 mM MOPS buffer, pH 7.0; 162.5 mM NaCl; 32.5% FSW), then transferred to (2) 1.25% glutaraldehyde in FB overnight at 4°C.

Specimens were washed once in FB without glutaraldehyde then three times in Tris-buffered Saline + Tween-20 (TBST: 0.1 M Tris buffer, pH 7.5; 0.15 M NaCl; 0.1% Tween-20). Proteinase K was applied for 10–15 min at 50 ng/μl in TBST. The digestion was stopped with 25 mM glycine in TBST, then specimens were washed two times in TBST and post-fixed for 30 min at room temperature with 4% paraformaldehyde in 50 mM MOPS buffer, pH 7.0 + 150 mM NaCl. Preceding hybridization, specimens were washed three times in TBST then transitioned into hybridization buffer (HB: 50% formamide; 5× saline–sodium citrate (SSC); 20 mM Tris-base, pH 7.5; 5 mM ethylenediamine tetraacetic acid (EDTA); 0.1% Tween-20; 2× Denhardt's Solution; 50 μg/ml Heparin; 500 μg/ml yeast tRNA) in three steps: 30% HB in TBST, 60% HB in TBST, then 100% HB. Specimens were incubated in HB for 1 hr at 60°C to prehybridize.

Digoxigenin (DIG) -labeled antisense probes were made by in vitro transcription using Sp6 or T7 RNA polymerase (New England Biolabs) with DIG RNA Labeling Mix (Roche, Indianapolis, IN) according to the manufacturer's protocol. Sense probes were used as negative controls. Probes were diluted to 1 ng/μl in HB and heated to 70°C for 5 min, then added to specimens for 12–16 hr hybridization at 60°C. Posthybridization washes included 15–20 min incubations at 60°C in the following solutions: HB; 50% HB + 50% 2× SSCT (2× SSC + 0.2% Tween-20); 2× SSCT; 0.5× SSCT; 0.2× SSCT. Specimens were then returned to room temperature, transferred to clean wells, and washed three times in TBST.

Specimens were blocked for 30 min at room temperature with 10% sheep serum + 1 mg/ml bovine serum albumin (BSA) in TBST. Anti-digoxigenin-AP, Fab fragments from sheep (Roche) were added in a 1:1,000 dilution in 5% sheep serum + 1 mg/ml BSA in TBST, then incubated for 1 hr at room temperature. Specimens were washed three times in TBST, then two times in alkaline phosphate buffer (APB: 100 mM Tris-base pH 9.5; 100 mM NaCl; 50 mM $MgCl_2$; 1 mM levamisole; 0.1% Tween-20). Stain was developed in 0.3375 mg/ml NBT + 0.175 mg/ml BCIP in APB for 1–24 hr depending on the probe and transcript abundance. Reactions were quenched with 50 mM EDTA in TBST. Specimens were transitioned into 50% glycerol in TBST with 5 mM Na-azide, and then photographed with a Canon EOS 60D camera (Canon, Lake Success, NY) through a ×40 air (0.75NA) Neofluar objective on a Zeiss Axiovert S100 microscope.

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