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Review

EXPERT OPINION

The importance of drug transporters in human pluripotent stem cells and in early tissue differentiation

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Introduction: Drug transporters are large transmembrane proteins which catalyse the movement of a wide variety of chemicals, including drugs as well as xeno- and endobiotics through cellular membranes. The major groups of these proteins include the ATP-binding cassette transporters which in eukaryotes work as ATP-fuelled drug 'exporters' and the Solute Carrier transporters, with various transport directions and mechanisms.

Areas covered: In this review, we discuss the key ATP-binding cassette and Solute Carrier drug transporters which have been reported to contribute to the function and/or protection of undifferentiated human stem cells and during tissue differentiation. We review the various techniques for studying transporter expression and function in stem cells, and the role of drug transporters in foetal and placental tissues is also discussed. We especially focus on the regulation of transporter expression by factors modulating cell differentiation properties and on the function of the transporters in adjustment to environmental challenges.

Expert opinion: The relatively new and as yet unexplored territory of transporters in stem cell biology may rapidly expand and bring important new information regarding the metabolic and epigenetic regulation of 'stemness' and the early differentiation properties. Drug transporters are clearly important protective and regulatory components in stem cells and differentiation.

Keywords: ABC transporters, drug transporters, human stem cell differentiation, pluripotent stem cells, regulation of transporter expression, SLC transporters

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1. Introduction

According to our recent understanding, most of the cellular substrates are taken up and extruded by membrane-bound transporter proteins, while passive diffusion through the cell membrane has a relatively limited role in these processes. However, hydrophobic compounds, including drugs, may rapidly enter the cell membrane by diffusion, and specific protective transporters are required to preserve cell integrity against such agents. Drug and xenobiotic transporters are large transmembrane proteins which catalyse the movement of a wide variety of chemicals, including drugs, as well as xeno- and endobiotics through cellular membranes. The major groups of these proteins include the ATP-binding cassette (ABC) transporters which in eukaryotes work as ATP-fuelled drug 'exporters' and the Solute Carrier (SLC) transporters, with various transport directions and mechanisms.

In this review, we summarize the recent knowledge about the ABC and SLC drug transporters in human pluripotent stem cells which are one of the most

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promising sources for cell therapy in the future. As detailed in this paper, the techniques for accurately measuring the expression, localization and function of drug transporters in stem cells may require special considerations, since in many cases, the relatively low levels of a selected transporter may be fundamentally important under various stages of human cell differentiation. A major part of this review is focusing on the presentation of the somewhat contradictory results concerning ABC transporter expression, highlighting the expression and role of the ABCG2 multidrug transporter, which seems to have an important protective effect in the undifferentiated stem cells, but also has a greatly variable expression during further tissue differentiation. In another section, we review the relatively small and scattered amount of data regarding SLC transporter expression and function in stem cells. In this case, we know a lot more about the function of these transporters in the placenta and in the differentiated tissues, while a thorough examination of the SLC transporter expression pattern in the undifferentiated human stem cells is still missing.

In the final section of this review, we discuss the regulatory pathways modulating xenobiotic transporter expression during the pluripotent state and early differentiation. Interestingly, several complex pathways, from the regulation of transcription, translation and the post-translational modifications, significantly affect drug transporter expression and localization. We feel that covering this relatively unexplored territory of transporters in stem cell biology may call the attention to the metabolic and epigenetic regulation of 'stemness' by drugs and xenobiotics, as well as these effects on early stem cell differentiation properties.

2. Techniques for studying transporter expression in human stem cells

Drug transporters are often rapidly regulated both at the transcriptional and translational levels to allow efficient protection of the cells from toxic environmental conditions [1] or, as in the case of ABCG2, also to regulate cell cycle progression.[2] Therefore, varying levels of protein expression can be expected not only at the population level, but even when following a single cell in time. Accordingly, the assays used for studying transporter expression should be sensitive enough to detect even low endogenous expression levels. In addition, examinations – whenever possible – should target both the mRNA and the protein expression levels and preferably also assess the functionality of the transporters.

Most of the techniques for studying transporter expression in human stem cells were taken over from the field of cancer research, studying ABC transporter-mediated multidrug resistance (MDR) in cancerous tissues or cell lines. Heterogeneous and low-level expression of transporters found in clinical samples called long ago for standardization of the measurement techniques.[3] However, in spite of the various measuring techniques accumulated over the decades in this field, some problems in the proper assessment of transporter expression are still present in the area of human stem cell research.

An example for these controversial findings is the case of ABCG2 multidrug transporter expression in hPSCs. Previous findings showed that ABCG2 expression is a conserved feature of tissue stem cells in the bone marrow and in many other solid organs [4,5]; therefore, the question arose whether hPSCs express this transporter. Several different answers were provided, ranging from no expression [6] to the detection of ABCG2 mRNA and its translation to functional protein [7] (more on this topic in Section 3). Several explanations were suggested to resolve these differences, for example that insufficient culture condition might promote expression of the functional ABCG2 transporter or that a long 3′-untranslated regions (UTR) variant of the ABCG2 mRNA might be a target for miRNA-based repression of translation, as found in some hESC lines.[8] In addition to potential biological causes, technical issues might also play a role in the described phenomenon. Cancer cell lines usually extremely overexpress the MDR transporters as a result of long-term drug selection or artificial gene delivery; therefore, techniques detecting transporter expression in these types of systems may fail to be sensitive enough to detect low levels of endogenous transporter expression, possibly occurring in hPSCs.

In this part of the review, we summarize the most relevant techniques and share our experience regarding the pitfalls of studying transporter expression in human stem cells and how to avoid them. We also emphasize that standardization of protein detection protocols – especially in the case of ABCG2, where function-dependent conformational changes of the ABCG2 can modify its interaction with the often used monoclonal antibody [9,10] – should provide more comparable results. After this standardization, the involvement of specific biological mechanisms can be properly explored, even considering the potential cell line or culture condition differences.

2.1. Studying transporter expression at the mRNA level

Several methods allow the detection of transporter mRNA expression levels including microarrays and quantitative real time PCR (qRT-PCR) measurements, as elegantly reviewed by Gillet *et al.* [11]. These methods are especially relevant when a wide range of transporter expression profile of the cells has to be examined, since detection of numerous human transporters at the protein level is hindered by the lack of reliable antibodies. However, the sensitivity of these methods varies as the extensive comparison by Gillet and colleagues showed.[11,12] While microarrays proved to be less adequate to discriminate between products of highly homologous genes, the Human ABC Transporter TaqMan Low-Density Array (TLDA) has been documented to be most sensitive and best suited for accurately quantitating individual ABC transporter genes in a high-throughput format.[11,12]

Therefore, when assessing the transporter profile of hPSCs and early derivatives, currently the TLDA should be the method of choice.[13]

ABC transporter mRNA levels significantly change over the course of hPSC differentiation [13,14]; therefore, parallel to the examination of transporter profiles, a pluripotency array analysis should also be performed to validate the pluripotent state of the hPSCs. Pluripotency arrays usually contain pluripotency and lineage-specific markers, allowing the identification of hPSCs as pluripotency-positive/lineage-negative cells and also their differentiated progenies, becoming pluripotency negative/lineage positive. Thus, characteristic transporter mRNA expression patterns for different cell types at various maturation stages can be investigated in an isogenic system.[13,15]

Findings of Rottenberg and colleagues [16,17] indicate that even a low-level increase in the mRNA levels of ABC transporters can be relevant and translate to transporter levels which are able to protect tumour cells against chemotherapy. In their studies, drug-sensitive and drug-resistant tumour samples showed less than 10-fold difference for ABCB1 and less than 5-fold for ABCG2 mRNA levels. As these elevations of mRNA expression can result in biologically relevant functional membrane protein expression, an important point is to follow actual transporter expression, localization and function.

2.2. Studying transporter protein expression and localization

The currently employed key techniques in this regard involve immunocytochemistry and flow cytometry analysis. However, in many cases, regular fluorescence microscopy may not be sensitive enough for the detection of low levels of immunofluorescence, which is characteristic for the low-level endogenous transporter expression in stem cells, as compared to transporter overexpressing cancer cell lines. Still, excitation with the adequate laser lines and detection with photomultiplier in most cases allows the sensitive examination of immunostaining of endogenous transporters in hPSCs. Laser-based flow cytometry measurements provide a sensitive method for the detection of endogenously expressed, immunolabelled transporters, and in addition, this method provides quantitative results.

In addition of choosing sensitive detection systems, the staining protocols should also be optimized for each and every antibody and detection method. In general, to maximize signal over background ratio, a bright fluorochrome (e.g. phycoerythrin) should be used for the secondary antibody labelling when antigens are expected to be represented at low density. Whereas flow cytometry applications are less affected, the poor photostability of phycoerythrin must be considered when signal detection involves microscopy applications with high-intensity illumination, long exposure times or repetitive image acquisition from the same area of the sample. In addition, to increase the detection of monoclonal primary antibodies, subclass-specific secondary antibodies should be preferably used.[18]

The case of ABCG2 (and the 5D3 monoclonal antibody raised against it) is again a good example how improved interaction between antigen and antibody can enhance the sensitivity of detection. During the transport cycle, the conformation of ABCG2 changes. In intact cells, as shown by Özvegy-Laczka *et al.*, the cell surface epitope recognized by the 5D3 monoclonal antibody exhibits an ABCG2 conformation-dependent accessibility.[9] Treatment with the ABCG2 inhibitor Ko143 or ATP depletion can stabilize ABCG2 in a specific conformation that increases 5D3 binding and results in an improved immunofluorescence signal.[10] Figure 1 shows the effect of Ko143 on 5D3 binding of ABCG2 on HUES3 hESCs detected by flow cytometry. In the case of endogenous expression levels, applying 'tricks' like these can make the difference between detectable and non-detectable presence of transporters in intact cells. It is important to emphasize, however, that the 5D3 antibody is only one among the antibodies featuring conformation-dependent binding characteristics, as similar conformation-dependent binding was reported in the case of the ABCB1-detecting antibodies, MRK16 [19] and UIC2 [20]).

An important question is the localization of the transporter proteins. In many cases, drug transporters can perform their original function only when localized to the cell surface, while during their production or membrane reshuffling, they temporarily reside in intracellular membrane compartments. Since several monoclonal antibodies react with intracellular epitopes of the transporter proteins, for immunodetection, the cells have to be permeabilized, and a proper distinction between plasma membrane (PM) or intracellular membrane localization of a given transporter is not an easy task. In fact, PM and intracellular membrane transporter expression profiles can only be distinguished when specific organelle (membrane) markers are co-stained with the transporters, and microscopy-based applications are used for detection. When antibodies recognizing cell surface epitopes are available, these allow the investigation of intact cells and offer an easy way for the quantitative assessment of cell surface localization, for example by flow cytometry. Figure 2 shows ABCG2 staining in intact HUES3 embryonic stem cells with the 5D3 antibody, recognizing cell surface epitope and, in parallel, the BXP-21 and BXP-34 antibodies, recognizing intracellular epitopes as well in fixed HUES3 cells. A similar labelling feature obtained by these antibodies suggests that in HUES3 cells, the expression of ABCG2 might be restricted to the PM. However, the absence of intracellular localization should be investigated by microscopy-based detection techniques as well.

2.3. Studying transporter functionality

While most of the transporter substrates are either toxic to or metabolized by the transporter expressing cells, relatively

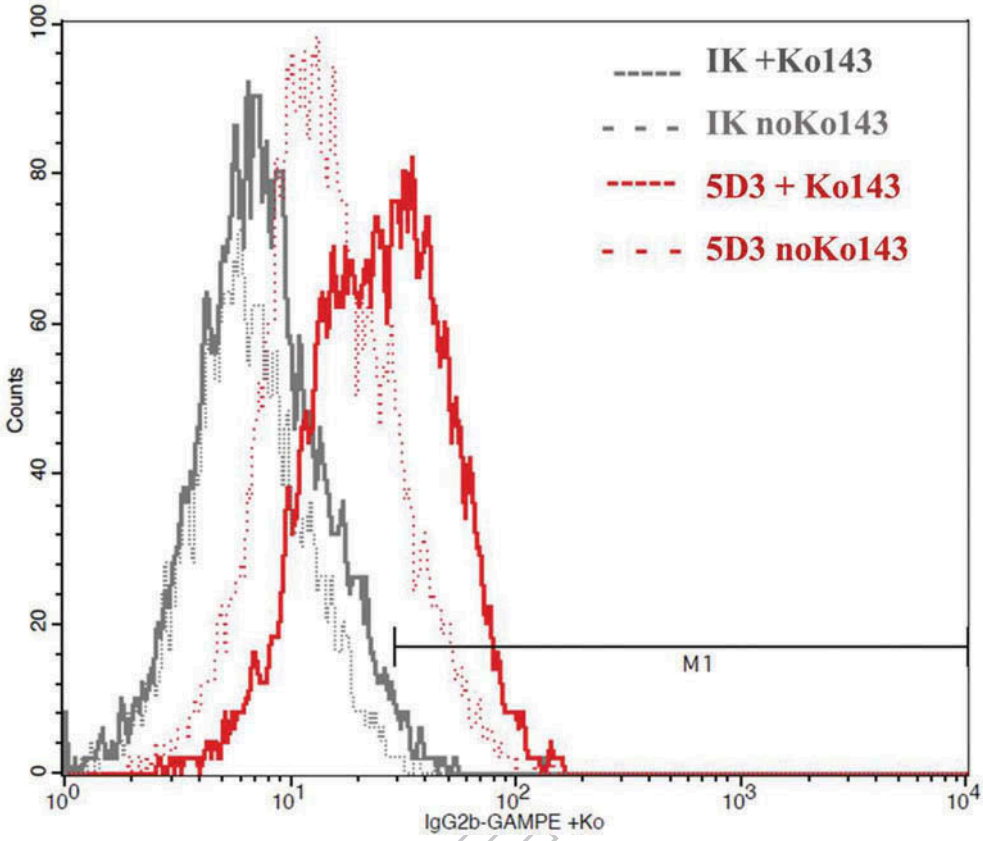

Figure 1. Indirect staining of ABCG2 with 5D3 monoclonal antibody, with or without the addition of ABCG2 inhibitor Ko143 in intact HUES3 hES cells. GAM-PE was used as secondary antibody, IK: isotype control.

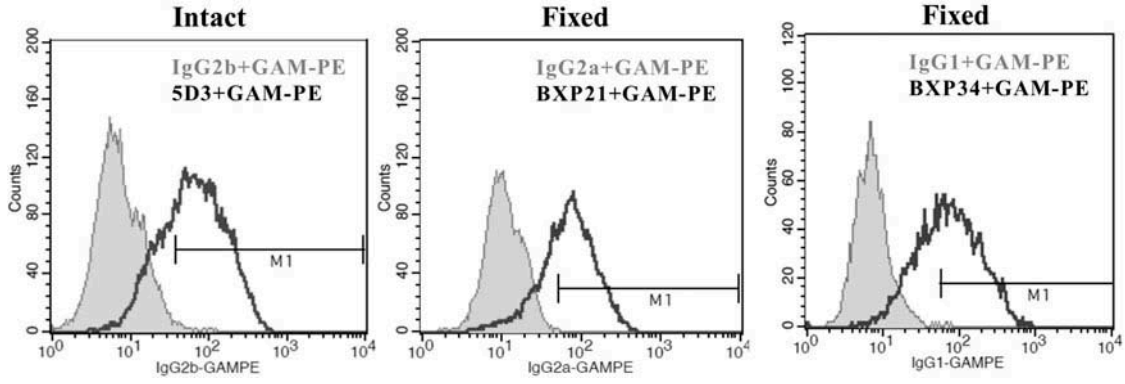

Figure 2. Indirect staining of ABCG2 with 5D3 monoclonal antibody (+Ko143) in intact hESC cells, as compared to Bxp21 and Bxp34 antibody staining in fixed HUES3 hES cells. GAM-PE was used as secondary antibody, and corresponding IgG primary antibodies were used as isotype control.

inert fluorescent dyes are most suitable for directly assessing drug-related transporter activity. By now, cellular or vesicular uptake and related fluorescence measurements are widely applied, and for the three major ABC multidrug transporters (ABCG2, ABCB1 and ABCC1), several fluorescent substrates have been described and successfully used in the literature.[21,22] Some of the indicator dyes (e.g. rhodamine 123, 3,39-diethyloxacarbocyanine iodide (DiOC2(3)), calcein-AM, chloromethylfluorescein diacetate (CMFDA), Pheophorbide A) are transported by one or two of the three

transporters, while mitoxantrone is a substrate of all three proteins.[21,22] However, point mutations can alter substrate specificity as it was shown for doxorubicin and rhodamine 123 in the case of ABCG2.[23,24] Recently, novel fluorescent probes (Green and Gold eFluxx-IDH® MDR probes), which are substrates of ABCB1, ABCG2 and ABCC1 transporters, have been suggested to assess low-level transporter expression and function.[25] For measuring SLC transporter activity, an array of fluorescent substrate molecules has also been developed (see [22]). The fluorescent compounds used for the uptake measurements may be substrates for more than one transporter, and the effect seen might be the result of synergistic transport mechanisms. Therefore, the use of selective inhibitors is highly recommended, while application of weak or nonspecific inhibitors (e.g. verapamil for ABCG2) should be avoided.[26,27]

The first functional identification of tissue-derived cells with stem cell-like properties was achieved by a lower level accumulation of the Hoechst 33342 dye in such cells, forming a 'side population'.[28,29] It has been documented that the active extrusion of this dye by the ABCG2 protein is responsible for this phenotype,[30] while other ABC transporters may also be relevant in Hoechst 33342 extrusion.

For assessing functionality of ABC-MDR transporters, the frequently used fluorescent dyes (CalceinAM, mitoxantrone, Hoechst, or DCV) provide a strong fluorescent signal, and uptake assays can be carried out by flow cytometry or microscopy-based techniques (e.g. fluorescence and confocal microscopy or high content screening) when assessing cells with high-level transporter expression. Mostly two samples are compared during the investigation: one contains cells incubated with a transporter-specific inhibitor and the fluorescent substrate, while the second sample is only incubated with the fluorescent dye. The fluorescence signal in the two samples is detected as an end point assay or by following time-course of the uptake, and functionality is determined based on the difference of the two signals.

In cases when drug transporters are present only in low levels, only kinetic measurements of the dye uptake are appropriate to assess functionality.[7] End point measurements (as applied by Zeng and colleagues [6]) may fail to provide appropriate information about functionality and expression, since low levels of endogenously expressed transporters will not be able to prevent dye accumulation in the presence of a huge amount of dye in excess for extended periods of time. Therefore, end point measurements may show much lower transporter levels than immunolabelling or kinetic studies (see [7,13,31,32]).

Kinetic dye transport measurements can be carried out by flow cytometry, as 'continuous flow' measurements.[33,34] Figure 3 shows a mitoxantrone uptake measurement in HUES9 hES cells (upper panel) and the effect of the

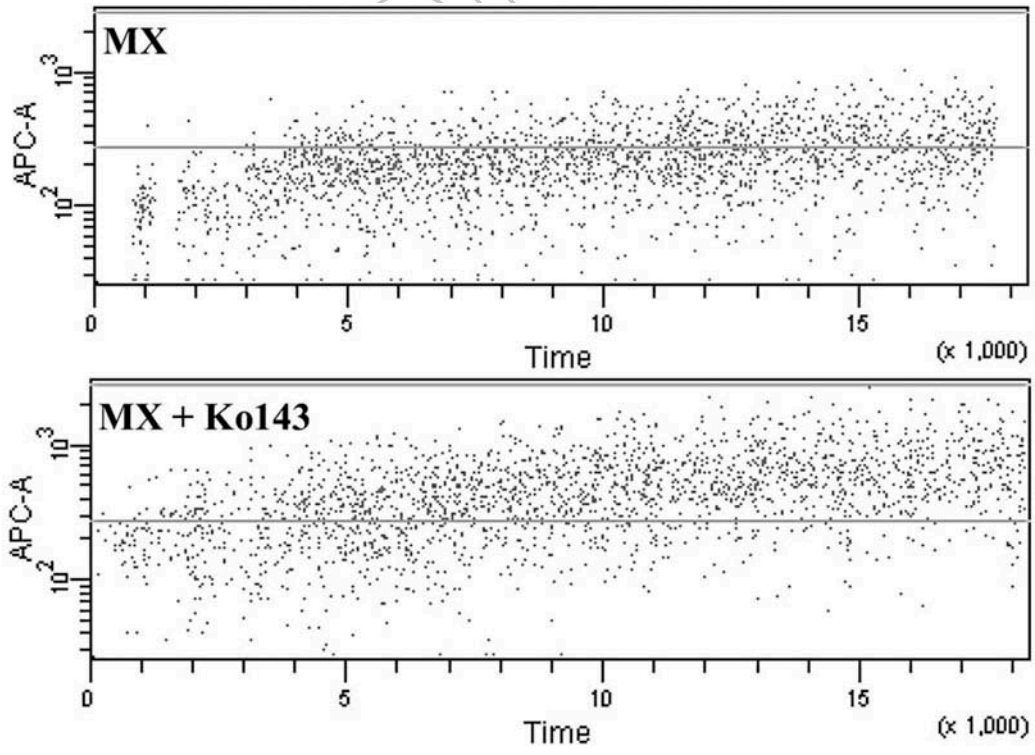

Figure 3. Kinetic transport measurement of mitoxantrone (100 nM) with or without inhibition of ABCG2 by Ko143 (5 μM) in HUES9 hESC cells. Each tick of the time parameter represents 10 ms, the duration of the measurement was 3 min.

Ko143 ABCG2 inhibitor (lower panel). A clear shift in the mitoxantrone signal is detectable in the presence of Ko143 as compared to mitoxantrone treatment alone, indicating that this method allows the assessment of functionality even when transporters are expressed at low levels. An important issue is to reduce the excess dye to the lowest possible level to render the transport process detectable; in our experience, the use of 100 nM of mitoxantrone allows the detection of the signal by flow cytometry. However, a high number of cells is needed for this type of measurement, which significantly hampers the feasibility of this method in the case of hPSCs.

In pluripotent stem cells, transporter expression may be heterogeneous. As shown by us [7] and others,[8] heterogeneity in hESC cultures regarding ABCG2 expression is manifested in a way that some colonies express ABCG2, while others do not, although the amount of colonies staining positive for ABCG2 shows high variability (see below in Section 3). Therefore, to achieve the highest possible sensitivity, the kinetics of the fluorescent dye uptake has to be selectively analysed in ABCG2-positive and -negative cells (colonies). This can be done by confocal laser scanning microscopy measurements, where differences in Hoechst dye uptake kinetics of ABCG2-positive and -negative cells can be investigated simultaneously. The inhibition of the dye uptake by selective inhibitors should also be employed to accurately identify the transporter involved in dye extrusion. For example, when treated with the selective ABCG2 inhibitor Ko143, an increased dye uptake can be observed in ABCG2-positive hPSCs, while the time course of the dye uptake in ABCG2 negative cells is not affected appreciably by the inhibition of the transporter.[7] It has to be mentioned that the specificity of Ko143 for ABCG2 is concentration dependent, since Ko143 at higher concentrations has an effect on both ABCB1 and ABCC1 transport activity.[35,36]

In addition to direct transport measurements, the ability of the endogenously expressed transporters to protect the cell population from effects of drugs and xenobiotics in long-term studies should also be examined. In these experiments, the goal is to find the range of drug concentrations at which the transporter-expressing pluripotent cells can survive, while the inhibition of the transporter results in the eradication of this cell population. This concentration can be quantitated by viability assays designed for high-throughput fluorescence or absorbance measurements.[37] In addition, the examination of pluripotency markers during these types of experiments also have to be performed, since some hPS cells may survive the drug treatment or toxic stress, but respond with rapid cell differentiation.[31]

3. Expression of ABC transporters in human embryonic stem cells and in early differentiation

ABC membrane proteins play a major role in pharmacokinetic variability of many drugs; therefore, they are intensively investigated in human tissues and tumours.[38] Moreover, the expression profiles of ABC transporters could be also important in performing stem cell-based cell therapies. Still, until now, there are only three detailed studies published about ABC transporter expression in pluripotent stem cells. All these studies used TLDA for the determination of mRNA levels of the ABC proteins.

Tang *et al.* [39] analysed a large amount of data by comparing 11 hematopoietic stem cells (HSCs) with six unrestricted somatic stem cells, seven mesenchymal stem cells, three multipotent adult progenitor cells, and three ESC lines (namely HUES1, HES2, and HES3). All the somatic stem cell samples originated from different donors, such as the embryonic stem cells. The authors found that each type of stem cells showed a unique ABC transporter profile while these profiles clustered together. Non-HSCs exhibited rather similar ABC transporter expression profiles, suggesting that in ABC transporter expression, the tissue specificity is a more important determinant than the genetic background. In all stem cells, the mRNA for ABCA1, ABCB6, ABCB8 and ABCD3 were found to be appreciably expressed. Regarding hESCs, the expression of ABCA3, ABCA7, ABCC8 and ABCG2 mRNAs were significantly higher, while ABCA8 and ABCC9 mRNAs were much lower (about 1000-fold lower) than in the non-HSC types. As a summary, this study provided a great deal of information about the expression of ABC transporters in various types of stem cells and highlighted the importance of tissue specificity.

The work from Barbet *et al.* [40] presented the ABC protein mRNA expression profiles of three hESC lines (H7, hES2 and hES3), two hESC-derived MSCs and six patient-derived MSCs, by using TLDAs. They found that most of the ABC proteins (44 from 48) were expressed in hESCs, although some of them showed significant but low-level gene expression. Their data confirmed that hESC-derived MSCs represent an intermediate state between hESCs and patient-derived MSCs, according to ABC protein expression. They also found mRNA of ABCE1, ABCF1-3, ABCB6,7,8,10, ABCC1,10 and ABCD3 appreciably expressed in all cell types, similarly to that shown by Tang *et al.* This analysis demonstrated that the mRNA of PM transporters showed the most variable expression levels among the ABC proteins. Based on this finding, they suggested an important role of ABC membrane transporters in pluripotency, and this work demonstrated that the ABC protein mRNA expression profile can serve as a tool for identification of the developmental state of the samples.

Erdei *et al.* [13] used an isogenic system to characterize the differentiation status of stem cells, based on ABC protein mRNA expression levels. HUES9 hES cells and their derivatives (cardiac, neural and mesenchymal cells) were investigated. After the detailed characterization of pluripotency and differentiation status of the hESC-derived cell types (using TLDA for Human Stem Cell Pluripotency), they studied the

mRNA expression of ABC transporters by TLDA for ABC transporter genes. The results showed a strong correlation between ABC protein expression and the developmental status of the samples, that is the more differentiated tissues clustered distinctly from those of the hESC samples. ABCE1, ABCF1-3, ABCB6,7,8,10, ABCC1,4,5 and ABCD3,4 mRNAs were appreciably expressed in all samples, similarly to that found in the work of Barbet *et al.* This paper also showed that ABCG2 mRNA expressed at a significantly higher level in hESCs than in the differentiated derivatives. For validation of mRNA data, the expression of the most significant ABC transporter proteins (ABCA1, ABCB1, ABCB6, ABCC1, ABCC6 and ABCG2) was also directly analysed by FACS and confocal microscopy, by using reliable specific antibodies. The protein expression showed a close correlation to the mRNA data.

An important issue is the potential expression of the ABCG2 multidrug transporter in stem cells. ABCG2 expression and the related side-population characteristics were shown earlier as a marker for tissue-derived stem cells,[4,5] and in earlier studies, this expression was also used as a marker of 'stemness' in pluripotent stem cells.[41–43] The mRNA expression of ABCG2 was demonstrated by RT-PCR in human embryonic cell lines H1,[42] BG01 and BG02,[43] and GE01, GE09, BG01, BG02, and TE06,[41] while the protein expression was demonstrated only in mouse cell lines.[41] As a next step, our group confirmed the mRNA expression by qRT-PCR and showed the ABCG2 protein expression by using 5D3 antibody in flow cytometry and confocal microscopy in the human pluripotent embryonic stem cell lines, HUES1, HUES3, HUES4 and HUES9.[7,13,32] The ABCG2 transporter showed PM localization and heterogeneous distribution on population level in all studied cell lines. Moreover, the function of ABCG2 was demonstrated by a kinetic microscopy measurement of Hoechst dye uptake in undifferentiated HUES9 cells. This uptake, reduced by ABCG2 protein function, was modulated (the dye uptake increased) by a specific ABCG2 protein inhibitor.[7]

Following these observations, controversial findings were published by Zeng *et al.*, showing no expression of ABCG2 in several pluripotent cell lines, including the embryonic stem cells H9, HUES1 and CT2 and two human iPS cell lines, iPS(IMR90)-1 and iPS(foreskin)-1.[6] The investigation of the mRNA levels by RT-PCR and studying ABCG2 protein expression by monoclonal antibody staining and Hoechst dye uptake did not show the sign of ABCG2 expression. Meanwhile, the differentiated stem cell derivatives towards trophoblasts showed all the signs of ABCG2 expression.

In contrast, the experiments by Padmanabhan and colleagues confirmed the ABCG2 mRNA expression in 9 hESC lines (BG01, BG02, BG03, ES02, SA01, TE03, UC01, UC06, WA01 and WA09), while they found no ABCG2 protein expression by Western blot analysis. When WA01 and WA09 cells were differentiated towards trophoblast direction by adding BMP4 to the culture media, an elevated ABCG2 expression was observed both at the mRNA and protein levels, while BG03 cells showed no sign of ABCG2 expression after BMP4 treatment.[8]

A further study by Hirata et al.[14] showed well measurable ABCG2 expression at the mRNA level in the hESC lines KhESC-1, KhESC-2, KhESC-3, KhESC-4 and KhESC-5, as well as in three hiPSC lines (201B7, IMR90-1 and IMR90-4). These authors also found appreciable ABCC1 mRNA expression in the undifferentiated cells, while detected no expression for ABCB1 and ABCC2. When the pluripotent cells were treated by retinoic acid, together with the appearance of the CDX2, a differentiation marker for placenta, ABCG2 and ABCB1 expressions also significantly increased.

In Table 1, we summarized the results obtained in the literature regarding ABCG2 expression. The inconsistent results might come from different genetic background of the cell lines studied, a lack of sensitivity of the detection methods (see Section 2.1 above), different forms of cellular states or stress conditions, suboptimal growth conditions and different handling of the hESC cultures in vitro. These may include the different growth media with various growth factors, extracellular matrices, passaging methods, and so on.

As suggested by Padmanabhan *et al.* [45], the observed differences in ABCG2 expression may also result from the variable regulation of ABCG2 at all the levels, including transcriptional, post-transcriptional, translational and/or post-translational levels. In addition, developmental changes significantly alter the stem cell ABC protein expression profiles [13,39,40]; thus for any transporter studies, it is crucial to exactly determine the differentiation status of the cell population examined.

The results for ABCG2 expression are more consistent when relatively pure hPSC-derived, partially differentiated cell types were analysed. MSCs differentiated from hESCs did not express ABCG2 either at mRNA [13,40] or at the protein level.[7,13] Similar results were found examining ABCG2 expression in ES-derived cardiomyocytes.[13,14] Differentiation towards trophoblasts showed an opposite effect on ABCG2 mRNA and protein expression. Stem cell differentiation into trophoblast direction, induced by BMP4 [8,43] or retinoic acid,[14] significantly increased ABCG2 expression. On the other hand, controversial results were obtained when ABCG2 mRNA expression was investigated in various spontaneous stem cell differentiation models.[6,7,13,14] This variability may be the result of the emergence of various differentiated subpopulations with different ABCG2 expressions during such a process.

Despite some contradictory results regarding ABCG2 expression levels and distribution in undifferentiated pluripotent stem cells, there are convincing studies about the important role of this multidrug transporter in the defence of pluripotent cells against harmful effects. When studying the effect of hypoxia/reoxigenation in BG01 and H9 pluripotent ES cell lines, Das and colleagues [44] found the emergence of an ABCG2+/SSEA3+ population which

Table 1. Summary of the results obtained in the literature regarding ABCG2 expression.

Cell line	Array	RT-PCR	IC	FACS	WB	Hst	Refs
iPS 201B7 (MEF)		Yes					Hirata [14]
BG01 (MEF)		Yes *	No		No		Padmanabhan [8]
BG01 (MEF)				Yes			Das [44]
BG01 (MEF)		Yes					Zeng X [43]
BG01 (MEF)		Yes					Bhattacharya [41]
BG02 (MEF)		Yes *					Padmanabhan [8]
BG02 (MEF)		Yes					Zeng X [43]
BG02 (MEF)		Yes					Bhattacharya [41]
BG03 (MEF)		Yes *	No				Padmanabhan [8]
CT2 (MG)		No	No		No	No	Zeng H [6]
E0S2 (MEF)	Yes						Barbet [40]
ES02	Yes						Tang [39]
ES02 (MEF)		Yes *					Padmanabhan [8]
ES03	Yes						Tang [39]
ES03 (MEF)	Yes						Barbet [40]
GE01 (MEF)		Yes					Bhattacharya [41]
GE09 (MEF)		Yes					Bhattacharya [41]
HUES1	Yes						Tang [39]
HUES1 (MEF)		Yes *	Yes	Yes		Yes	Apáti [7]
HUES1 (MG)		No	No		No	No	Zeng H [6]
HUES3 (MEF)		Yes *	Yes	Yes			Erdei [13]
HUES4 (MEF)		Yes *	Yes	Yes			Erdei [13]
HUES4 (MEF)		Yes *	Yes	Yes			Sarkadi [32]
HUES9 (MEF)	Yes		Yes	Yes			Erdei [13]
HUES9 (MEF)		Yes *	Yes	Yes			Apáti [7]
iPS IMR90-1 (MEF)		Yes					Hirata [14]
iPS IMR90-1 (MG)		No	No		No	No	Zeng H [6]
iPS IMR90-4 (MEF)		Yes					Hirata [14]
iPS(FS)-1 (MG)		No	No		No	No	Zeng H [6]
KhESC-1 (MEF)		Yes					Hirata [14]
KhESC-2 (MEF)		Yes					Hirata [14]
KhESC-3 (MEF)		Yes					Hirata [14]
KhESC-4 (MEF)		Yes					Hirata [14]
KhESC-5 (MEF)		Yes					Hirata [14]
SA01 (MEF)		Yes *					Padmanabhan [8]
TE03 (MEF)		Yes *	Yes				Padmanabhan [8]
TE06 (MEF)		Yes	Yes				Bhattacharya [41]
UC01 (MEF)		Yes *					Padmanabhan [8]
UC06 (MEF)		Yes *					Padmanabhan [8]
WA01 (MEF)		Yes					Ginis [42]
WA01 (MEF)		Yes *	No		No		Padmanabhan [8]
WA07 (MEF)	Yes						Barbet [40]
WA09 (MEF)				Yes			Das [44]
WA09 (MEF)		Yes *	No				Padmanabhan [8]
WA09 (MG)		No	No		No	No	Zeng H [6]

Cell lines are listed according to their NIH codes. The abbreviations of the techniques are array: TaqMan® ABC gene Arrays (TLDAs); IC: immunocytochemistry; Hst: Hoechst dye uptake measurements; and WB: Western blot; '*' stand for quantitative RT-PCR. 'Yes' indicates when ABCG2 expression was found, 'no' indicates when ABCG2 expression was under the detection level and empty spaces were used when data are not available.

showed 'enhanced stemness' phenotype, that is decreased p53 expression and elevated HIF-2alpha, OCT4 and NANOG expression, and enhanced cytoprotective features both in vitro and in vivo. As demonstrated by Erdei *et al.* [31] in HUES9 embryonic stem cells, the functional presence of ABCG2 provides important protection against various harmful conditions, including physical stress, drugs and UV light exposure. The stress-induced production of metabolites could induce genetic and/or epigenetic changes (such as DNA methylation) which could potentially remove the cells from the carefully balanced state of pluripotency and induce differentiation. ABCG2 could potentially remove such stress-induced metabolic compounds, thus protecting the stem cells. Although only a few systematic studies have been published on this topic, they point towards an important protective role of ABCG2 in various stem cells, potentially operating not only at the cellular but also at the population level.[31,44]

4. Solute carrier (SLC) transporters in human embryonic stem cells and in tissue differentiation

SLCs are solute transporting integral membrane proteins, named and grouped based on functional criteria, while sequence homology between the members of this large superfamily (comprising of almost 400 different transporters grouped into 52 families) is small or nonexisting.[46–51] Most SLC proteins contain large transmembrane domains with typically 10–12 alpha helices and catalyse the transmembrane movement of diverse molecules, ranging from large organic anions to organic cations, ions and gases. Many forms of transport, from unidirectional movement to obligatory co-transport or coupled exchange, are catalysed by the various SLC proteins. Within the 52 families, based on structural, evolutionary, functional and sometimes historical basis, the proteins are named as SLC + family number + letter of subfamily + number of isoform (e.g. SLC2A1, a glucose exchange transporter).

In many cases, SLC transporters are localized to the PM and their function is to promote the uptake of cellular nutrients, endobiotics and signalling molecules; therefore, they are often denoted as 'uptake transporters'. Still, the function of numerous SLCs also involves the removal of toxic endo- and xenobiotics, the regulation of pH or to move molecules between intracellular compartments (see [46,50]).

Currently, we have little information regarding the role of SLC transporters in pluripotent stem cells and a little more in early cell differentiation. In the huge comprehensive review collection, detailing the structure, function and disease-connections of all groups of SLC transporters in 2013 (see [52]), there is hardly any mentioning of stem cell connections, and in the following years, this information has also been very limited. Since SLC transporters can serve both as drug targets and mechanisms to facilitate or reduce drug delivery to stem cells and differentiating tissues, the exploration of their role in these cell types should be of utmost importance.

Specific nutrient transporters, for example the isoforms of the SLC2 (PM, sugar, mostly glucose) and SLC3 (PM, mostly amino acid) transporters, serve as markers during stem cell differentiation, since differentiated tissues show major changes in their expression levels and patterns. As examples, in the fertilized egg, undifferentiated stem cells or preimplantation embryos, the major glucose transporter is SLC2A1 (GLUT1), while in many differentiated tissues SLC2A2, A3 or A4 take over this role. In induced and directed hepatocyte, enterocyte or blood–brain epithelial cell differentiation from embryonic stem cells, in addition to ABC transporters (see above), several SLC-type transporters are important developmental indicators [1,15,53–60]. During stem cell differentiation into early kidney tissues, the expression patterns of the SLC12A1 (NA/K/Cl – thick ascending limb the of Henle's loop), A3 (Na/Cl – distal convoluted tubule) and the SLC34A1 (Na/phosphate, proximal tubule) transporter proteins can be efficiently used as markers for kidney tubule development [61]. Similarly, specific substrate transporters in the SLC1 (PM glutamate, aspartate or other neurotransmitter carriers), in the SLC6 (PM neurotransmitter and amino acid transporters) and in the SLC17 family (vesicular glutamate transporters) are recognized as regulated marker proteins, especially in neuronal differentiation.[62] An isoform of the PM Na-H exchanger (SLC9) proteins, SLC9C1, is selectively expressed in spermatozoa; SLC11A2 (a metal transporter) and SLC19A2 (a thiamin transporter) are essential in erythroid progenitor cells, while SLC16A13 (a PM proton-linked monocarboxylate transporter) is almost exclusively localized in bone marrow stem cells (see [63,64], and [52,65]).

The SLC transporters having important role in drug and xenobiotic transport include the family of SLCO (formerly SLC21) PM organic anion transporters, the SLC22 PM zwitterion and organic cation and anion transporters, and the SLC47 (MATE, Multidrug and Toxin Extrusion) a family of PM drug transporters. In the following section, we review the potential role of these proteins in stem cells and early differentiation.

The human SLCO (formerly SLC21) family of Organic Anion Transporting Polypeptides (OATPs) comprises of 11 PM transmembrane proteins, with variable transport mechanisms. Many SLCO transporters are preferentially expressed in the liver, kidney and the blood–brain barrier and are involved in the transport of amphipathic, anionic and even cationic compounds of endogenous (e.g. steroids, prostaglandins, bile acid etc.), or exogenous (e.g. cholesterol lowering statins, antibiotics and anticancer drugs) origin. OATPs may influence the pharmacokinetics of several clinically relevant drugs and evoke complex drug–drug interactions.[66]

The pattern of OATP expression (particularly SLCO1B1 and 1B3) has been already used to follow early hepatic, intestinal and blood–brain barrier epithelial cell differentiation from pluripotent stem cells.[15,67] While in foetal tissues, the OATP levels are low, fully differentiated hepatocytes have high expression levels for selected OATPs, thus report the success of end-stage hepatocyte differentiation.[57] OATPs have also been used to characterize potential cancer stem cells.[68–70] Since the uptake of various anticancer drugs (e.g. methotrexate, taxol, flavopiridol or imatinib) is mediated by OATPs, their increased expression in some cancer tissues, as compared to normal cells, may help to devise suitable tumour therapies.[71]

The 13 members of the SLC22 family are PM transporters for organic cations (OCTs), organic cations and zwitterions (OCTNs) and, in the case of some members, organic anions (OATs). These proteins contain 12 transmembrane helices, catalyse facilitated diffusion (OCTs, OCTNs), anion exchange (OATs), or Na+/zwitterion co-transport (OCTNs). SLC22 proteins are major players in intestinal absorption and hepatic and renal excretion of drugs, xeno- and endobiotics (including neurotransmitters, e.g. acetylcholine, cAMP, cGMP or prostaglandins), and are also functionally expressed

in neuronal and cardiac tissues. SLC22A1-3 (OCTs) are involved in the uptake of numerous therapeutic agents, including metformin, antiviral drugs or irinotecan, as well as in cisplatin toxicity in cancer, while SLC22A12 (URAT1) is a major uric acid transporter in humans.[72]

There are again very few data for the function of the SLC22 type transporters in stem cells – these proteins are significantly expressed only during tissue differentiation. Interestingly, in murine embryonic stem cells, the inhibition of acetylcholine release through organic cation transporters significantly affects cellular homeostasis, indicating that non-neuronal acetylcholine and its release through OCTs may have a functional role in embryonic stem cells.[73] SLC22A4 (OCTN1) is widely expressed in numerous tissues and relatively highly expressed in mesenchymal stem cells and neuronal progenitor cells (NPCs). This protein was found to be a transporter for ergothioneine (ERGO), a zwitterionic natural antioxidant, which seems to have an important physiological role in NPCs.[74] OCTN1-mediated uptake of ERGO in NPCs inhibits cellular proliferation, while promotes neuronal differentiation by modulating the expression of basic helix–loop–helix transcription factors.[75]

Hormones and transcription factors in the process of tissue differentiation from embryonic stem cells significantly modulate the expression of SLC22A1 (OCT1), SLC22A2 (OCT2), SLC22A6 (OAT1) and SLC22A8 (OAT3). Although with practically unknown function, SLC22A18 is expressed at relatively high levels in adult and foetal kidney and liver and in differentiated kidney proximal tubules. Mutations of the SLC22A18 gene cause rhabdomyosarcoma and are associated with lung cancer.[72]

Interestingly, SLC22A2 (OCT2), SLC22A3 (OCT3) and SLC22A18 are among the not too many (in this case maternally) imprinted genes in the hESCs, and the expression levels of these proteins are greatly increased during EB formation and further tissue differentiation.[76] This strong developmental regulation also alters their imprinting status, resulting in variable monoallelic and biallelic expression patterns in a lineage-specific manner,[77] which may be important in developmental physiology or disease development.

The SLC47A1 and A2 (MATE1 and 2) proteins are PM efflux transporters mediating the H+-dependent excretion of organic cations in various epithelial tissues. MATEs are multispecific transporters and export a number of endogenous organic cations as well as drugs, including cimetidine, metformin, guanidine, procainamide, cephalexin or cephradine. The driving force for organic cation extrusion by MATEs is provided by the oppositely directed proton gradient. In humans, MATE1 is highly expressed in the kidney and liver, MATE 2 preferentially in the kidney, and both proteins are localized at the apical membrane of proximal tubules and hepatocytes.[78,79] Human MATE1 is also detectable in other tissues, including adrenal gland, skeletal muscle, testis and the placenta.

A relatively more detailed information is now available for the presence and role of SLC transporters in the feto–maternal complex tissue of the human placenta. Between the first and third trimester, there are major alterations in the trophoblast SLC expression patterns: SLC2A3/GLUT3, SLC22A3/OCT3, SLC38A5 and SLCO4A1/OATP4A1 are highly expressed in the first trimester and decrease during gestation, while the expression of SLCO2B1 shows an opposite pattern.[80] The syncytiotrophoblasts and the cytotrophoblast also show different expression patterns for several transporters. MATE1 is expressed at the maternal side of the trophoblast cells in the placenta in the first trimester, while its expression becomes negligible in the term placenta, thus this transporter may have a protective, toxin elimination function for the foetus in early gestation stages.[67] Interestingly, some of the SLC transporters, which play a key role in the neurotransmitter transport and the pharmacological treatment of anxiety and depression, may have an important role in drug abuse during pregnancy.[81] Cocaine and amphetamine by inhibiting the placental SLC transporters cause harmful effects, such as premature delivery or growth retardation, by elevating serotonine and norepinephrin concentrations in the intervillous space. Also, a decreased expression of SLC22A3/OCT3 may be involved in preeclampsia.[82]

5. Regulation of ABC transporter expression – focus on ABCG2

Our knowledge on gene expression regulation at the RNA level are constantly becoming far more complex than previously appreciated, due to the unprecedented speed of data generated by the next generation sequencing (NGS) methodologies,[83] and projects aiming to decipher potential functions of the non-protein coding regions of the genomes, such as the ENCODE project.[84,85] By now, the complex regulatory network of transcriptional and splicing regulation is joined by the equally complex network of non-coding RNAs and post-transcriptional regulatory players, most prominently the microRNAs (miRNAs).[86] To various levels and contributions, these regulatory processes seem to operate on the expression of every gene, and drug transporters are no exception from these. The translational and post-translational regulation of membrane transporters are very complex, and this might have been the reason why the RNA regulation of the corresponding genes gained much less attention until recently. Nevertheless, more and more data point out that in certain cell types, such as in various stem cells, the fine-tuning of transporter regulation at the RNA level plays a crucial role in transporter function, often having medical relevance, as in the case of multidrug transporters.

There are numerous studies describing the transcriptional regulation of drug transporters in various cell lines, although very few data are available about these mechanisms in stem cells. Since extensive reviews are available on these topics,[21,87] the detailed description of this regulation is

beyond the scope of the present review. In the following sections, we highlight some aspects of mRNA regulation of transporters, especially the importance of the noncoding, UTRs in membrane protein expression that must be integrated into the complex picture of transporter regulation.

5.1. Alternating the 5′UTR – regulation by transcription and splicing

5′UTRs can regulate the translation efficiency from the mRNA by various mechanisms: by upstream open reading frames they can interfere with the translation from the canonical start codon; by upstream alternative translation start sites (AUGs, GUGs, etc.), they may produce longer protein isoforms; or alternatively, 5′UTR binding factors can regulate the stability of the transcript itself, resulting in lower amount of the corresponding protein.[88] In addition, one gene might produce more alternative 5′UTR sequences, either by distinct transcriptional start sites or alternative splicing, with the potential of distinct regulation of the different mRNA molecules and, therefore, of the encoded protein species. A prominent example is the ABCG2 multidrug transporter, where at least 3 different noncoding leader exons exist (both in human and in mouse cells), producing different 5′UTR containing mRNAs but encoding for the same protein species.[89,90]

Apart from having medical relevance in cancer therapy, this gene is expressed in embryonic stem cells[7,91] and was proven to be the molecular basis for the so-called side population phenotype.[30] An intriguing observation is that only two of the 5′UTR variants (having exon 1a and 1b) are expressed in human embryonic stem cells, whereas the third variant (with exon 1c) is only present in dendritic cells of hematopoietic origin.[7] The ABCG2 5′UTR pattern is different in various cell types, but certain drug-selected cell lines also express the 1a and 1b variants similarly to stem cells.[7,90] The need of high ABCG2 protein levels in those cell types could be achieved by the usage of alternative transcriptional start sites: certain signal transduction pathways seem to distinctly regulate the exon 1a and exon 1b containing transcripts.[92] Nevertheless, the role in translation regulation of the different 5′UTRs could also be important, and the presence of certain variants might represent selective advantage for the cells by more efficient translation of this multidrug transporter (Sándor S et al., manuscript in prep.)

Until now there have been relatively few studies on transporter mRNA 5′UTR regulation in stem cells, but some key examples for membrane proteins highlight the importance of investigating this level of regulation. The mouse and the human folate receptor-alpha membrane proteins have been shown to be upregulated during retinoic acid-induced differentiation of embryonic stem cells, producing a distinct pattern of various 5′UTR containing transcripts.[93] The exact functional differences of the mRNA variants remain to be elucidated but the evolutionary conservation indicates their significance. A more detailed characterization of the human PTCH1b transmembrane protein expression pattern revealed a complex regulation via alternative 5′UTRs: upstream open reading frames motifs, upstream AUG codon producing an N-terminally longer protein and internal ribosomal entry site (IRES)-like sequences as cis-regulatory elements can increase the membrane protein repertoire, especially under certain stressful conditions, including hypoxia.[94] In addition, polymorphisms in 5′UTR sequences might also be associated with complex phenotypes, such as birth weight in the case of solute carrier family 44 variants.[95] The mechanisms by which the functions of different 5′UTRs are manifested are often not clear as yet, and this indicates the need of further studies aiming to specifically investigate these regulatory levels for membrane transporters.

5.2. Regulation by the 3′UTR – the miRNA network

The regulatory role of the 3′UTRs are also well known: length and sequence of this region influence mRNA stability, translation efficiency and often mRNA localization inside the cell.[96] In recent years, it became evident that at least half of the human protein-coding genes have variable 3′UTR containing mRNAs, produced by alternative cleavage and polyadenylation.[97] A striking observation was that this phenomenon strongly influences membrane protein expression and localization: when studying the role of alternative 3′UTRs, Berkovits and Mayr found that a long 3′UTR sequence increases the efficiency of cell surface expression of CD47 membrane protein.[98] It turned out that the sequence exerts its function by recruiting the HuR and SET proteins to the site of translation, and their interaction with the freshly translated cytoplasmic domain of CD47 results in the translocation of CD47 to the PM. This trafficking of different 3′UTR mRNA variants by the HuR protein also affects the cell surface expression of other membrane proteins, and it could be a widespread mechanism by which transporter protein localization and function could be regulated without changing the coding region of the gene. Since the HuR protein have been shown to bind numerous mRNAs,[99] this regulatory mechanism could influence other important drug transporters, potentially involved in early differentiation processes from stem cells.

A prominent pathway by which the role of 3′UTRs is manifested is the miRNA regulatory pathway. miRNAs are small, non-coding species binding to these sequences, and initiating the translational repression and/or the decay of the target mRNAs.[86] One miRNA could target several mRNA species and vice versa, one particular mRNA 3′UTR generally contains several different miRNA binding sites. By this complex regulatory network, the exerted effects of miRNAs are comparable to that of transcription factors, playing important roles in all aspects of cellular physiology. As such, drug transporters are also under the influence of miRNA regulation. The ABCG2 multidrug transporter, being expressed in human embryonic stem cells (see above,

part 5.1), has also different 3′UTR variants produced by alternative mRNA cleavage and polyadenylation. We could show earlier that only the shorter version is detected in undifferentiated human embryonic stem cells, which can therefore escape the regulation of several miRNAs, the binding sites of which are missing from this shorter 3′UTR.[7] The physiological significance of this regulation was provided by independent studies: drug resistant cancer cell lines express shorter 3′UTRs of ABCG2 mRNA, which enables them to escape the negative regulation by certain miRNAs, including hsa-miR-328 and hsa-miR-519c.[100,101] In addition, this phenomenon should be considered when new alternative ABC transporter or membrane protein inhibitors are being developed, as the miRNA-based novel products may not work efficiently for different tumours.[102,103] These examples underline the need of studies aiming to decipher the role of miRNA regulation in drug transporter expression; apart from understanding cellular mechanisms of these subtle mRNA regulations, these molecular details would definitely have medical importance in the future.

6. Conclusion

Drug transporters play an important role in protecting human pluripotent stem cells and their differentiated progenies against toxic materials. These large transmembrane proteins catalyse the movement of a wide variety of chemicals, including drugs as well as xeno- and endobiotics through cellular membranes. The major human drug transporter groups include the ABC transporters, working as ATP-fuelled drug 'exporters' and the SLC transporters, with various substrate transport directions and mechanisms. Both ABC and SLC drug transporters may serve as markers for a certain stages of stem cell differentiation, but also seem to have a function in supporting and regulating 'stemness' and early cell differentiation, especially in the adjustment to environmental challenges. The current amount of information regarding the expression and role of drug transporters in human stem cells is relatively limited, thus this unexplored territory of transporters may rapidly expand in the near future.

7. Expert opinion

The protection of pluripotent stem cells and their differentiated progenies against toxic environmental factors has an utmost importance in early human development. Hydrophobic compounds, including drugs and xenobiotics, in many cases rapidly enter the lipophilic cell membranes, and specific membrane transporter proteins are required to modulate the final cellular drug levels, thus protecting our cells against these compounds. SLC transporters, sometimes denoted as 'uptake transporters', in most cases facilitate the transcellular clearance of drugs, with a number of variable transport mechanisms. In contrast, ABC transporter proteins work as ATP-dependent primary active drug extrusion pumps. All these drug and xenobiotic transporters are large transmembrane proteins which catalyse the movement of a wide variety of chemicals with a relatively promiscuous pattern of interactions.

In this review, we summarize and discuss the available data for the key ABC and SLC drug transporters which contribute to the protection of undifferentiated human stem cells and the cells during initial tissue differentiation. Many of these transporters have a relatively low level of endogenous expression; therefore, we present the major methodological problems and discuss some solutions for properly assessing transporter expression, localization and function in stem cells. We demonstrate the great challenge of properly analysing transporter expression and function in the continuously changing forms of stem cells between pluripotent and partially differentiated states. We especially focus on the regulation of transporter expression by factors modulating cell differentiation properties. In addition, we also present available data for the function of drug transporters in adjustment to environmental challenges.

Although some early studies had conflicting results due to methodically questionable measurements, based on the currently available, data we can conclude that some of the ABC transporters, especially the ABCG2 multidrug transporter, have a key role in the protection of undifferentiated human stem cells against toxic materials and stress conditions.

In addition, numerous other ABC transporters are differently expressed and regulated during early stem cell differentiation, which may have a functional consequence in the drug sensitivity and other transport properties of these newly generated cell types. We know much less about SLC transporter expression in pluripotent stem cells, while a larger amount of information is available for the foetal and placental human tissues in this regard.

As a summary, the area of drug transporters and their role in pluripotent stem cells is an as yet relatively unexplored territory of cell biology. Altogether this research has a great potential to help our understanding of the complex responses of stem cells to environmental challenges. Based on the current surge of studying human embryonic and induced pluripotent stem cells, we may expect a rapid expansion of this knowledge, which may also help us to understand the regulation and toxic modulation of 'stemness' and early differentiation.

Article highlights

- The key drug transporters in human stem cell membranes are ABC and SLC transporters.
- Human pluripotent stem cells variably express membrane drug transporters, which provide protection against toxic compounds and environmental challenges.
- The ABCG2 protein may serve as a stem cell marker and is a key protective transporter.
- Methodological problems are crucial in properly determining stem cell transporter expression.

- Drug transporter expression patterns significantly depend on culturing conditions and early cell differentiation.

This box summarizes key points contained in the article.

Declaration of interest

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