Cytometry: Part B - Clinical Cytometry

Expression pattern of the human ABC transporters in pluripotent
embryonic stem cells and in their derivatives

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Expression pattern of the human ABC transporters in pluripotent embryonic stem cells and in their derivatives

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Abstract

Background: ATP-binding Cassette (ABC) transporters have key roles in various physiological functions as well as providing chemical defense and stress tolerance in human tissues. In this study we have examined the expression pattern of all ABC proteins in pluripotent human embryonic stem cells (hESCs) and in their differentiated progenies. We paid special attention to the cellular expression and localization of multidrug transporter ABC proteins.

Methods: Stem cell differentiation was carried out without chemical induction or cell sorting, and specialized cell types were separated mechanically. Cellular features regarding pluripotency and tissue identity, as well as ABC transporter expression were studied by flow cytomtery, immuno-microscopy and qPCR-based low-density arrays.

Results: Pluripotent hESCs and differentiated cell types (cardiomyocytes, neuronal cells and mesenchymal stem cells) were distinguished by morphology, immunostaining markers and selected mRNA expression patterns. We found that the mRNA expression levels of the 48 human ABC proteins also clearly distinguished the pluripotent and the respective differentiated cell types. When multidrug and lipid transporter ABC protein expression was examined by using well characterized specific antibodies by flow cytometry and confocal microscopy, the protein expression data corresponded well to the mRNA expression results. Moreover, the cellular localization of these important human ABC transporter proteins could be established in the pluripotent and differentiated hESC derived samples.

Conclusions: These studies provide valuable information regarding ABC protein expression in human stem cells and their differentiated offspring. The results may also help to obtain further information concerning the specialized cellular functions of selected ABC transporters.

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Introduction

The ABC protein family is present from bacteria to humans, and the 48 human ABC proteins include several transporters, channels, as well as non-membrane proteins (1). During the past decades a large amount of information became available about the expression and function of these human proteins, while there are still numerous unresolved questions in this field. We have shortly summarized the recent knowledge about the human ABC proteins in **Supplementary Table 1.**

Certain ABC transporters form a special network of chemo-defense system, as these ATP-dependent active transporters extrude a wide variety of substrates from the cells, including endo- and xenobiotics. While these ABC transporter (with a key role of ABCB1/Pgp, ABCC1/MRP1 and ABCG2/BCRP) proteins play an important role in protecting our body, they are also involved in causing multidrug resistance in cancer cells (2,3). One member of these multidrug transporters, ABCG2, has been shown to play a major role in the protection of stem cells against toxic compounds. In addition, the ABCB6 protein has been indicated to play a major role in the defense against toxic heme derivatives, while the ABCA1 protein has a key role in the extrusion of excess cellular lipid derivatives, especially cholesterol (see refs (4-9)).

Since the human embryonic stem cells (hESC) require special protection during development, and some of the differentiated tissues are also well protected against toxic agents, an important task is to follow the changes in the expression and localization of the ABC multidrug transporters during these early developmental processes.

In our previous studies we have shown that the HUES9 embryonic stem cells express the ABCG2 protein at the cell surface (10), and this transporter has an important role in defending the HUES9 cells during stress conditions (5). In the current work we have examined the full pattern of 48 human ABC protein mRNA expression levels by qPCR-based microarray, in order to provide information about changes in their tissue expression patterns in undifferentiated and selectively differentiated hES cells. We have also followed the expression of selected ABC transporter proteins by flow cytometry and immunostaining during early human cell differentiation. In addition to provide basic information about ABC protein expression, these studies also allowed to examine the cellular localization of these transporter proteins which was not exactly known for embryonic stem cells and their derivates.

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Materials and Methods

Cell culture and differentiation

The hES cell line HUES9 (originally provided by Dr. Douglas Melton, Harvard University) were maintained on mitotically inactivated mouse embryonic fibroblasts (MEF) and spontaneous differentiation were performed via embryoid body (EB) formation as described previously (11). After 6 days embryoid bodies were placed onto gelatin coated 24 well plates, where they attached to the surface and underwent spontaneous differentiation. The desired cell types were separated as follows:

Some of the EB-outgrowths start spontaneously beating within a few days post-plating. These rhythmically contracting areas were isolated mechanically and hES cell-derived cardiomyocytes were re-plated on gelatin coated 8 well confocal chambers or harvested to RNA isolation. Neuronal progenitor cells with rosette-like morphology were mechanically isolated for generation of hES cell-derived neural cells, between days 8 and 10. These rosettes were able to re-attach onto gelatin coated surfaces and continue their further differentiation to mature neural cell types. The RNA isolation and immunostaining were performed at day 24.

The mesenchymal stem cell like (MSCl) cells were generated and maintained as described previously (12). For more details see the Supplementary Methods.

Flow cytometry

Single cell suspensions were prepared by gentle trypsinization, and the cells were labeled in PBS containing 0.5% bovine serum albumin with appropriate antibodies. In all hESC samples an anti-mouse Sca-1 (Ly-6A/E) (FITC or PE, BD Pharmingen) antibody was employed for gating out the positively labeled mouse feeder cells.

We used the following directly labeled anti-human antibodies: SSEA4-APC and PODXL-PE (R&D Systems) for investigation of pluripotency. For indirect staining of ABC transporters the following monoclonal antibodies were applied: 5D3 (BD Pharmingen) for ABCG2, MRK16 (Alexis Biochemicals) for ABCB1, anti-Lan (OSK43 (generated and kindly provided by Hideo Takahashi Japanese Red Cross Osaka Blood Center, Japan) (13)) for ABCB6, R1 (Abcam) for ABCC1 and AB H10 (Abcam) for ABCA1 labeling. The 5D3 labeling was performed in the presence of Ko143, a specific inhibitor of ABCG2, which maximizes 5D3 binding (14). Control staining with appropriate isotype-matched control mAbs was included. For fixation and permeabilization of the cells the Fix&Perm (Invitrogen) solution was used, according to the manufacturer's instruction. Samples were analyzed by a BD FACSCantoII flow cytometer (Becton Dickinson Immunocytometry Systems [BDIS]). For more details see the Supplementary Methods.

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Immuno-cytochemical staining

Immunostaining of all cell types was performed as described previously (10), except the cell surface labeling of ABCG2 which was carried out as described by (5). For labeling of pluripotent and differentiation markers the following primary antibodies were applied: Oct4 (SantaCruz), Nanog, SSEA4 and PODXL (RnD Systems) cardiac Troponin-I (Sigma), Nestin (Abcam), β-III Tubulin (RnD Systems) and CD-44-FITC (BD Pharmigen). The ABC transporters were studied by the same antibodies used for flow cytometry. Hoechst33342 (Invitrogen) was used for nuclear staining. The stained samples were examined by an Olympus FV500-IX confocal laser scanning microscope. For more details see the Supplementary Methods.

Gene expression analysis

Total RNA was isolated from the cells using the Trizol reagent (Life Technologies). RNA integrity was checked by standard gel electrophoresis, RNA concentration was determined by spectrophotometry using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). Gene expression profiles were determined by analysing TaqMan® Low Density Arrays (TLDA cards, Life Technologies) designed for mesasuring pluripotency marker genes (TaqMan® Array Human Stem Cell Pluripotency Panel, cat. #: 4385344) or ABC transporters (TaqMan® Array Human ABC Transporter Panel, cat. #: 4378700). Briefly, 500 ng of total RNA was used to prepare cDNA samples using the Reverse Transcription System Kit (Promega), according to the manufacturer's instruction. cDNA samples corresponding to 100ng of total RNA were combined with 2X TaqMan® Gene Expression Master Mix and loaded on one channel of the appropriate TLDA card; real-time PCR reactions were run on a 7900HT System according to the manufacturer's protocol (Life Technologies). For data analysis, the DataAssist™ Software v3.0 (Life Technologies) was used. Gene expression values were determined by the ΔΔCt method using multiple endogenous control genes on the TLDA cards (ACTB and GAPDH genes for the pluripotency array data and PRLP0 and PPIA genes for the ABC transporter array data). The average linkage clustering method was applied to analyze the expression data and to calculate Pearson's correlation values to define distances of our samples sets.

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Results

1. Differentiation of hES cells – characterization by differentiation markers

For studying the expression of human ABC proteins in pluripotent cells and in their differentiated derivatives, we have generated cardiac cells, mesenchymal-like stem cells (MSCls (12)) and neural cells from the HUES9 cells, by using a method of spontaneous differentiation, via EB formation **(Supplementary Fig. 1).** With this method the desired cell types could be generated from hESC without the addition of special chemicals, and the selection of the differentiated progenies was performed by enzymatic digestion and/or mechanical selection, without applying any drug selection or cell sorting. All the differentiated cell types were cultured in the same media and under similar conditions (see Materials and Methods). These uniform conditions were used because the expression levels and localization of several ABC proteins have been shown to be influenced by chemical inducers, selection drugs or antibiotics (see ref. (15,16)).

When selecting various tissue types, we performed a detailed phenotypic characterization of the parental and differentiated cell types, in order to assure that any further analysis should be performed by using properly selected populations. This characterization included the investigation of several cell type specific markers by flow-cytometry and immunocytochemistry.

As shown in **Figure 1A**, by flow cytometry studies we found that the pluripotent hES cells (HUES9) highly expressed the SSEA-4 and PODXL pluripotency markers on the cell surface. Using immunostaining and confocal microscopy, we could clearly show the presence of the nuclear pluripotency markers, Oct4 and Nanog, as well as the cellular membrane staining of SSEA-4 and PODXL **(Fig. 1B)**.

During differentiation studies, cell surface markers for MSCls were analyzed as described in detail in our previous work (see (12)). In all cases, immunostaining revealed proper separation of cells showing positive staining for representative markers of cardiac (cardiac troponin-cTNI), neural (neuron specific tubulin-β-III tubulin and Nestin) and mesenchymal (CD44) cell types (**Figure 1C).**

After this phenotypic selection and verification we have analyzed the mRNA expression patterns of the selected cell types by using a *TLDA pluripotency array*. Besides the differentiated cell types, the common progenies of the differentiated cells (mesenchymal, cardiac and neural) from 6 days old EBs were included in the further investigation. The array included 36 pluripotency, 19 mesoderm, 13 ectoderm, 17 endoderm and 4 trophectoderm markers, along with several housekeeping genes. An overview of these data (summarized in

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supplementary table 2) is demonstrated in a heat-map (**Fig. 2A**), showing relative gene expression levels. The cluster analysis (see Methods), documented in **Fig. 2B**, shows close correlation of the mRNA expression pattern with the differentiation status of the cell samples. As shown, the biological parallels (for hESC and MSCl) clustered together, while the undifferentiated hESC samples are clearly separated from the partially differentiated sample (6 days EB). The mRNA clusters of the differentiated cell types are noticeably different from that of the hESCs and from one another and the biological replicates of the most differentiated cell types (the MSCl cells, after 80 days of differentiation) are located away from the undifferentiated cells with the longest distance in the dendrogram. As shown by the highest expression levels found in the pluripotency array data, the hES cells showed high levels of the pluripotency mRNA markers, while the derivative cells showed upregulated levels of the proper lineage-specific markers (F**ig 2C)**.

2. ABC transporter expression in pluripotent and differentiated hES cells – flow cytometry studies

In these experiments we investigated the ABC transporter expression in the pluripotent and differentiated cells at the protein level. The application of specific and properly reactive antibodies is a crucial question in these experiments. Especially in the case of the flow cytometry studies, we used antibodies against extracellular epitopes to examine the expression and cell surface localization of ABCG2 (mAb 5D3), ABCB1 (mAb MRK16), and ABCB6 (mAb OSK43). However, most of the antibodies recognizing human ABC transporters have been generated against intracellular epitopes, and in several experiments we have used such antibodies (see Methods). In each case, the specific protein recognition was assured by examining various cell types overexpressing these ABC transporters (**suppl. fig 2a**).

The two major cell types in which ABC transporter expression could be studied without major cellular damage were the undifferentiated hES cells and the MSCs. In these cell types we focused on the expression of ABC multidrug or lipid transporters with key roles in cellular chemodefense and stress response. As shown in **Fig. 3,** in the case of ABCB1, ABCB6 and ABCG2 we used the respective antibodies recognizing extracellular epitopes, while in the case of ABCC1 and ABCA1 we employed antibodies generated against intracellular protein domains.

Fig. 3A shows flow cytometry detection of five ABC transporters (ABCB1, ABCB6, ABCG2, ABCC1 and ABCA1) in hESCs under non-permabilized conditions using antibodies recognizing external epitopes;, while **panel B** of Fig. 3 shows similar measurements after

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permeabilization of hESCs. As documented, in all cases we found a very low level expression in the non-permeabilized cells except ABCG2 which showed elevated level of cell surface expression, while a slight increase in the apparent expression of all these transporters, especially ABCA1 could be found in the permeabilized hESCs. Only ABCG2 expression level showed an apparent reduction under these conditions, showing that the 5D3 antibody binding was reduced by the applied fixation-permeabilization.

Fig. 4 documents similar studies carried out with hES-derived MSCls by flow cytometry. In this case the expression levels of all studied transporters were negligible without permeabilization (**Fig 4 A**), but were well detectable in the permeabilized cells in the case of ABCB6, ABCC1, and especially of ABCA1. It is important to note that ABCG2 expression was not detectable in these cells, with or without permeabilization.

In the case of ABCB1 and ABCB6 (antibodies reacting with external membrane epitopes) the potential expression of these proteins in intracellular membrane compartments may be suggested by these data. Since the ABCC1 and ABCA1 antibodies recognize intracellular epitopes, the increased protein levels found in the permeabilized cells most probably correspond to increased general expression levels, and in this case the plasma membrane or intracellular membrane expression levels cannot be distinguished.

3. ABC transporter expression in pluripotent and differentiated hES cells – immunomicroscopy studies

Flow cytometry examinations could not be performed with vulnerable cell types such as cardiomyocytes and neural cells, without injuring the cells. Comparative examination of the hESC and hESC derived cell types by immunohistochemistry is thus an additional method to characterize protein expression and yield more informative cellular localization data than flow cytometry.

In **Fig 5** we summarize the results of the immunostaining experiments. This figure compiles representative data for each cell type and for the above described antibodies recognizing selected ABC transporters. As shown in **Supplementary Figure 2B**, we have examined several selected cell types, overexpressing these ABC transporters, as positive controls (see also M&M).

As documented in **Fig. 5,** in the case of ABCA1 all hESC-derived cell types showed well measurable expression, and in the undifferentiated hESCs and the MSCls, the expression was homogenously high showing both plasma membrane and cytosolic localization. In contrast, only a small number of cardiac and neural cells showed ABCA1 expression, and this

expression was mostly localized in the cytosol. In the case of <u>ABCB1</u>, we found measurable expression only in the differentiated cardiac and neural cell types, which was in agreement with the flow cytometry data indicating no ABCB1 expression in the undifferentiated hESCs and the MSCls. The <u>ABCB6</u> protein was expressed ubiquitously in all of the examined cell types, and the expression was found in intracellular compartments. These data are again in line with the flow cytometry results which also indicated intracellular expression of this protein. For <u>ABCC1,</u> a well measurable expression could be observed for most of the cell types, except for the cardiac cells, showing a much lower protein level. In all cases, ABCC1 expression was detected in the plasma membrane compartment.

As shown earlier in **Fig. 3**, in the undifferentiated hES cells high level of ABCG2 protein expression was detected on the cell surface by flow cytometry and the immunostaining experiments shown in **Fig. 5** verified this phenomenon. In contrast, we could not detect a significant ABCG2 expression in the differentiated MSCl cell types and some expression could be detected on cardiac and neural cultures however this expression was confined to the border of tissues. In **Fig. 5,** we have also included immunostaining data for the ABCC6 protein, as this transporter has been indicated to play an important role in cardiac tissues (17,18). However, we could not detect ABCC6 expression in any of the examined hESC-derived cell types.

4. ABC protein mRNA expression in pluripotent and differentiated hES cells

Because of the lack of reliable antibodies and staining protocols for numerous human ABC proteins, a systematic analysis can only be performed for the respective mRNA expression levels. For this purpose we used a quantitative RT-PCR technology and ABC protein specific microarray, and examined the same mRNA samples as used in the pluripotency array analysis (see **Fig. 2**), obtained from pluripotent hESCs and the differentiated cell types. A visual overview of these gene expression data is demonstrated in a heat-map (**Fig. 6 A**), while the original Ct data are presented in the **Supplementary Table 3.**

When analyzing the ABC mRNA array we observed that the expression of ABC transporters in general showed lower mRNA expression levels than the pluripotency markers (average Ct (ABC) = 33,6 ± 2,8; average Ct (pluri) = 21,6 ± 7,6), as compared to the similar level measured for the expression of the housekeeping genes. Since membrane protein expression usually requires long term expression of relatively smaller amount of proteins, this result was in accordance with our expectations.

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Cluster analysis based on ABC protein expression patterns is shown in **Fig 6B**. Remarkably, clustering based on ABC transporter profiles was found to be similar to the hierarchy defined by pluripotency markers (see **Fig. 2**), indicating that all the examined cell types have characteristic expression patterns (fingerprints) for the ABC proteins, depending on the cell maturation status. As shown in **Fig 6C**, some of the ABC proteins showed similar (medium) level of expression in all cell types **(Fig 6C** left panel), although most of these ABC proteins are not well described transporters, and some are not even membrane proteins (see **Suppl. table 1**). These data suggest a role of these ABC proteins in the general maintenance of cellular homeostasis.

In the following analysis, we focused on the recognized ABC membrane transporters, and excluded those proteins which showed high Ct values, indicating extremely low or no expression (**Fig 6C** right panel). We compared the relative expression of ABC transporter mRNAs in differentiated cells to those in undifferentiated hES cells (**Supplementary table 4**).

As shown in **Fig7 and presented in supplementary table 4,** we found that most of the ABC transporters were expressed at significantly higher level (although in different magnitudes, see **Fig 7A and B**) in the differentiated cell types than in the pluripotent hESCs. When analyzing the cell-type dependent expression, we found that several ABC transporter mRNA levels showed large increase in differentiated progenies. These included the expression of ABCA8, ABCC3, ABCC9, and ABCG1. In contrast, there were several transporter mRNA expression levels characteristic for certain cell types: changes of ABCB1 and ABCC6 expressions were higher in cardiac cells and ABCA4 was higher in neural cells compared to hESC (**Fig 7A**). Note, that the only ABC transporter which showed a decreased expression level in all differentiated cell types was ABCG2 (**Fig 7B**).

This cell type-specific expression indicates a possible role of these proteins in the differentiated tissues and allows a further characterization of their physiological roles.

Discussion

ATP-binding Cassette (ABC) transporters play an important role in various physiological functions, maintain cellular homeostasis, and provide defense mechanism against toxic endo- and xenobiotics. These ABC transporters work in a general defense network system, having overlapping functions and substrate specificity. Based on their promiscuous recognition of toxic agents, they can functionally substitute each other. While many of the ABC multidrug transporters also have broad tissue distribution, their expression levels may provide a tissue-specific "fingerprint", corresponding to special metabolic or other cellular functions, as well as tolerance against stress and drugs.

In this study we have examined the protein level and cellular localization of the major multidrug transporters, as well as the mRNA expression pattern of all 48 ABC proteins in pluripotent human embryonic stem cells and in their selected, differentiated progenies. In order to investigate the human ABC transporter expression "fingerprint" in differentiating cells, we have used and characterized an experimental system involving four human cell types of isogenic origin. We have cultured human pluripotent hESCs, and in a spontaneous differentiation model generated well separable differentiated cell types, including cardiac cells, neural cells and MSC-like cells. The major advantage of this system is that the hES cells and the differentiated progenies have the same genetic background and cell differentiation did not involve major changes in the culturing conditions.

In these experiments first we examined pluripotency and tissue-specific marker protein expression in the selected cell types by flow cytometry and immunostaining. We have also carried out the investigation of the mRNA expression pattern of a wide range of pluripotency and differentiation markers by using RT-PCR based quantitative arrays. These studies established a proper characterization of the respective cell types.

When looking for ABC protein expression, we found that the mRNA expression levels of all the 48 human ABC proteins also clearly distinguished the pluripotent and the respective differentiated cell types. According to recent studies, distinct ABC protein patterns were observed when hESCs (HES2 and HES3) were compared to hematopoietic stem cells, unrestricted somatic stem cells and mesenchymal stem cells (19). It has also been shown that mRNA expression levels are different in hESCs and hESC-derived hMSCs (20). However none of these studies have investigated the ABC transporter expression at protein level. In order to perform a detailed characterization, we selected several key ABC transporters involved in multidrug resistance, providing defense against stress conditions, and modulating cellular lipid metabolism. These transporters were examined by using well characterized

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specific antibodies both in flow cytometry and confocal microscopy experiments. This way the cellular expression pattern and also the subcellular localization of these important human ABC transporters could be established in the pluripotent and differentiated hES cell derived samples. It is important to note that the obtained ABC transporter protein expression data closely corresponded to the respective mRNA expression results.

During our investigations, we found that the most prevalent multidrug transporter in the undifferentiated hES cells, in accordance with previous data, was the ABCG2/BCRP protein. This protein is expressed on the cell surface and, although this expression is heterogeneous, may significantly contribute to the defense mechanisms in pluripotent stem cells (sees refs. (10,19-22)). Another multidrug transporter found at low levels in the undifferentiated cells was ABCC1 (on the cell surface), while we could not detect ABCB1/MDR1 in this cell type. Two other ABC transporters found to be expressed in the hES cells were ABCA1, an important player in lipid/cholesterol extrusion, and ABCB6, a potential player in protecting against toxic heme derivatives. Interestingly, this transporter, noted to be also in the plasma membranes of various cells (23,24), showed only intracellular expression in all cell types examined here.

In the human ES-derived neural cells we observed high level expression for ABCB6 and ABCC1, and in certain cell regions for that of ABCA1 and ABCB1. In the cardiomyocytes high level expression of ABCB6 and lower levels for ABCB1 and ABCA1 were observed. Interestingly, certain cells in the external regions of cardiac and neural tissue samples also showed ABCG2 expression. In the MSCs a predominant, but mostly intracellular expression was observed for ABCA1, ABCB6 and ABCC1, while there was no measurable expression for ABCB1 or ABCG2. As of note, we did not find any significant ABCC6 expression at protein level in any of the cell types examined here.

As a summary, these studies may provide important information for a selective ABC protein expression pattern in human stem cells and in their differentiated offsprings. However, further studies are needed for the establishment of specialized inter-tissue and inter-organ distribution, as well as for the cellular functions of the key human ABC transporters during early human development.

Acknowledgments

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Disclosure statement

No competing financial interests exist.

References

1. Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. Genome Res 2001;11(7):1156-66.

2. Hyde SC, Emsley P, Hartshorn MJ, Mimmack MM, Gileadi U, Pearce SR, Gallagher MP, Gill DR, Hubbard RE, Higgins CF. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature 1990;346(6282):362-5.

3. Sarkadi B, Homolya L, Szakacs G, Varadi A. Human multidrug resistance ABCB and ABCG transporters: participation in a chemoimmunity defense system. Physiol Rev 2006;86(4):1179-236.

4. Ding XW, Wu JH, Jiang CP. ABCG2: a potential marker of stem cells and novel target in stem cell and cancer therapy. Life Sci 2010;86(17-18):631-7.

5. Erdei Z, Sarkadi B, Brozik A, Szebenyi K, Varady G, Mako V, Pentek A, Orban TI, Apati A. Dynamic ABCG2 expression in human embryonic stem cells provides the basis for stress response. Eur Biophys J 2012;42(2-3):169-79.

6. Krishnamurthy PC, Du G, Fukuda Y, Sun D, Sampath J, Mercer KE, Wang J, Sosa-Pineda B, Murti KG, Schuetz JD. Identification of a mammalian mitochondrial porphyrin transporter. Nature 2006;443(7111):586-9.

7. Hamza I, Dailey HA. One ring to rule them all: trafficking of heme and heme synthesis intermediates in the metazoans. Biochim Biophys Acta 2012;1823(9):1617-32.

8. Liu HF, Cui KF, Wang JP, Zhang M, Guo YP, Li XY, Jiang C. Significance of ABCA1 in human carotid atherosclerotic plaques. Exp Ther Med 2012;4(2):297-302.

9. Wenzel JJ, Piehler A, Kaminski WE. ABC A-subclass proteins: gatekeepers of cellular phospho- and sphingolipid transport. Front Biosci 2007;12:3177-93.

10. Apati A, Orban TI, Varga N, Nemeth A, Schamberger A, Krizsik V, Erdelyi-Belle B, Homolya L, Varady G, Padanyi R and others. High level functional expression of the

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ABCG2 multidrug transporter in undifferentiated human embryonic stem cells. Biochim Biophys Acta 2008;1778(12):2700-9.

11.   Orban TI, Apati A, Nemeth A, Varga N, Krizsik V, Schamberger A, Szebenyi K, Erdei Z, Varady G, Karaszi E and others. Applying a "double-feature" promoter to identify cardiomyocytes differentiated from human embryonic stem cells following transposon-based gene delivery. Stem Cells 2009;27(5):1077-87.

12.   Varga N, Vereb Z, Rajnavolgyi E, Nemet K, Uher F, Sarkadi B, Apati A. Mesenchymal stem cell like (MSCl) cells generated from human embryonic stem cells support pluripotent cell growth. Biochem Biophys Res Commun 2011;414(3):474-80.

13.   Helias V, Saison C, Ballif BA, Peyrard T, Takahashi J, Takahashi H, Tanaka M, Deybach JC, Puy H, Le Gall M and others. ABCB6 is dispensable for erythropoiesis and specifies the new blood group system Langereis. Nat Genet 2012;44(2):170-3.

14.   Ozvegy-Laczka C, Varady G, Koblos G, Ujhelly O, Cervenak J, Schuetz JD, Sorrentino BP, Koomen GJ, Varadi A, Nemet K and others. Function-dependent conformational changes of the ABCG2 multidrug transporter modify its interaction with a monoclonal antibody on the cell surface. J Biol Chem 2005;280(6):4219-27.

15.   Leslie EM, Deeley RG, Cole SP. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol Appl Pharmacol 2005;204(3):216-37.

16.   Takenaka K, Morgan JA, Scheffer GL, Adachi M, Stewart CF, Sun D, Leggas M, Ejendal KF, Hrycyna CA, Schuetz JD. Substrate overlap between Mrp4 and Abcg2/Bcrp affects purine analogue drug cytotoxicity and tissue distribution. Cancer Res 2007;67(14):6965-72.

17.   Kavukcuoglu NB, Li Q, Pleshko N, Uitto J. Connective tissue mineralization in Abcc6-/- mice, a model for pseudoxanthoma elasticum. Matrix Biol 2012;31(4):246-52.

18.   Mungrue IN, Zhao P, Yao Y, Meng H, Rau C, Havel JV, Gorgels TG, Bergen AA, MacLellan WR, Drake TA and others. Abcc6 deficiency causes increased infarct size and apoptosis in a mouse cardiac ischemia-reperfusion model. Arterioscler Thromb Vasc Biol 2011;31(12):2806-12.

19.   Tang L, Bergevoet SM, Gilissen C, de Witte T, Jansen JH, van der Reijden BA, Raymakers RA. Hematopoietic stem cells exhibit a specific ABC transporter gene expression profile clearly distinct from other stem cells. BMC Pharmacol 2010;10:12.

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20. Barbet R, Peiffer I, Hutchins JR, Hatzfeld A, Garrido E, Hatzfeld JA. Expression of the 49 human ATP binding cassette (ABC) genes in pluripotent embryonic stem cells and in early- and late-stage multipotent mesenchymal stem cells: possible role of ABC plasma membrane transporters in maintaining human stem cell pluripotency. Cell Cycle 2012;11(8):1611-20.

21. Sawicki WT, Kujawa M, Jankowska-Steifer E, Mystkowska ET, Hyc A, Kowalewski C. Temporal/spatial expression and efflux activity of ABC transporter, P-glycoprotein/Abcb1 isoforms and Bcrp/Abcg2 during early murine development. Gene Expr Patterns 2006;6(7):738-46.

22. Vieyra DS, Rosen A, Goodell MA. Identification and characterization of side population cells in embryonic stem cell cultures. Stem Cells Dev 2009;18(8):1155-66.

23. Kiss K, Brozik A, Kucsma N, Toth A, Gera M, Berry L, Vallentin A, Vial H, Vidal M, Szakacs G. Shifting the paradigm: the putative mitochondrial protein ABCB6 resides in the lysosomes of cells and in the plasma membrane of erythrocytes. PLoS One 2012;7(5):e37378.

24. Paterson JK, Shukla S, Black CM, Tachiwada T, Garfield S, Wincovitch S, Ernst DN, Agadir A, Li X, Ambudkar SV and others. Human ABCB6 localizes to both the outer mitochondrial membrane and the plasma membrane. Biochemistry 2007;46(33):9443-52.

25. Lee MN, Roy M, Ong SE, Mertins P, Villani AC, Li W, Dotiwala F, Sen J, Doench JG, Orzalli MH and others. Identification of regulators of the innate immune response to cytosolic DNA and retroviral infection by an integrative approach. Nat Immunol 2012;14(2):179-85.

26. Paytubi S, Wang X, Lam YW, Izquierdo L, Hunter MJ, Jan E, Hundal HS, Proud CG. ABC50 promotes translation initiation in mammalian cells. J Biol Chem 2009;284(36):24061-73.

27. Wanders RJ, Visser WF, van Roermund CW, Kemp S, Waterham HR. The peroxisomal ABC transporter family. Pflugers Arch 2007;453(5):719-34.

28. Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, Lagutina I, Grosveld GC, Osawa M, Nakauchi H and others. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 2001;7(9):1028-34.

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Figure Legends

Figure 1. Characterization of HUES9 cells and their derivates by following the expression of selected proteins.

(A) Investigation of the pluripotent state of HUES9 cells by flow cytometry. More than 90% of the cells show cell-surface SSEA4 and PODXL expression. M1: cell population with marker positivity was gated based on the relevant isotype-matched control mAbs.

(B) Investigation of the pluripotent state of HUES 9 cells by immunomicroscopy. HUES 9 cells were grown on MEF feeder cells for two days in eight-well chambers for confocal microscopy. Co-culture of HUES9 and feeder cells were fixed, permeabilized and stained with the antibodies recognizing the Nanog (red), Oct4 (green), SSEA4 (green) and PODXL (green) markers. Nuclei were counterstained with Hoechst33342 (blue). Nanog and Oct4 transcription factors showed nuclear localization, while SSEA4 and PODXL cell surface markers localized in plasmamembrane.

(C) Investigation of the differentiated forms of HUES9 cells by immunomicroscopy. HUES9-derived cell types were differentiated as described in Materials and Methods and were transferred mechanically into eight-well chambers for confocal microscopy. The samples were fixed, permeabilized and stained with antibodies recognizing specific proteins for each cell type; cTNI (green) for cardiac, β- III tubulin (green) and Nestin (red) for neural and CD44 (green) for mesenchymal cell type. Nuclei were counterstained with Hoechst33342 (blue).

Figure 2. TLDA analysis of differentiation status of HUES9 cells and their differentiated offspring

(A) Heat map representation of human pluripotency marker gene mRNA expressions in 7 samples of 4 cell types (hESCs a and b, cardiac, neural and MSCs a and b) and the 6 days old EB culture (the common progenitors of differentiated cell types). The pluripotency genes are shown on the y-axis, the 7 samples are ordered on the x-axis with biological replicates ("a" and "b"). The color code ranges from high (dCt = -6, light red) through medium (black), to low (dCt = 26, light green) levels of gene expression relative to selected housekeeping genes.

(B) Hierarchical clustering of 7 samples allowing a separation based on differentiation status of the samples.

(C) The highest gene expressions (5<Ct>15) for each cell type showing the proper characteristics for a given differentiation status.

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Figure 3. Flow cytometry analysis of ABC transporter expression in hES cells.

Single cell suspensions from HUES9 cells were obtained as described in Materials and Methods. Non-viable cells were gated out by 7AAD staining. Monoclonal antibodies specific for ABCB1, ABCB6, ABCG2, ABCC1 and ABCA1 were used to detect transporter expression (A) in intact cells (without fixation and permeabilization) and (B) in fixed and permebealized cells (for details see Materials and Methods). R1: cell population with marker positivity was gated based on the relevant isotype-matched control mAbs; MFI: mean fluorescence intensity.

Figure 4. Flow cytometry analysis of ABC transporter expression on MSCl cells.

Single cell suspensions from MSCl cells were obtained by gentle trypsinization. Non-viable cells were gated out by 7AAD staining. Monoclonal antibodies specific for ABCB1, ABCB6, ABCG2, ABCC1 and ABCA1 were used to detect transporter expression (A) on intact cells (without fixation and permeabilization) and (B) in fixed and permeabilized cells (for details see Materials and Methods). R1: cell population with marker positivity was gated based on the relevant isotype-matched control mAbs; MFI: mean fluorescence intensity.

Fig. 5 Immuncytochemical detection of ABC transporters in HUES9 cells and their derivatives.

HUES 9 cells were grown on MEF feeder cells for two days in eight-well chambers and HUES9-derived cell types were differentiated as described in Materials and Methods and were transferred mechanically into eight-well chambers for confocal microscopy. The samples were stained with specific antibodies to visualize ABCA1, ABCB1, ABCB6, ABCC1, ABCG2 and ABCC6 proteins (red) as described in the "Materials and methods" section. Nuclei were counterstained with Hoechst33342 (blue). Stained samples were examined by an Olympus FV500-IX confocal laser scanning microscope.

Figure 6. TLDA analysis of ABC protein gene expression in HUES9 cells and their differentiated offspring

(A) Heat map representation of human ABC protein gene expressions in 7 samples of 4 cell types (hESCs a and b, cardiac, neural and MSCls a and b) and the 6 days old EB culture (the common progenitors of differentiated cell types). The ABC genes are shown on the y-axis, the 7 samples are ordered on the x-axis with biological replicates ("a" and "b"). The color code ranges from high (dCt = 4, light red) through medium (black), to low (dCt = 14, light green)

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gene expression relative to selected housekeeping genes. (B) Hierarchical clustering of 7 samples allowing a separation based on differentiation status of samples. (C) The listed highest ABC gene expressions (27<Ct>32, bold) and lowest gene expressions (Ct>38, normal) were common for each cell type showing universal expression pattern for certain ABC proteins.

Figure 7. Relative gene expressions of ABC transporters in hESC derived cell types as compared to undifferentiated cells.

The relative gene expression levels of each transporter in hESCs are set at 1 and the fold differences in expression are presented on the graph.

Supplementary table 1. Summary of human ABC proteins

For more information see refs (http://humanabc.4t.com/main.htm, (9,25-28))

Supplementary table 2. List of pluripotency gene expression data

The Ct values are given for each cell type. Pluripotency markers (black), mesoderm markers (green), ectoderm markers (blue), endoderm markers (orange), trophectoderm markers (brown), housekeeping genes (italic, black), transcription factors (bold).

Supplementary table 3. List of ABC protein gene expression data

The Ct values are given for each cell type. The value of 40 indicates the lack of detection/expression of the corresponding mRNA.

Supplementary table 4. ABC transporter expression changes during differentiation towards cardiac, neural and mesenchymal cell types.

Supplementary Figure 1. Differentiation of hES cells via embryoid body system

An outline of the protocol used for the differentiation of human ESCs to cardiac, neural and mesenchymal cell types.

Supplementary Figure 2. Immunostaining for detection of ABC transporters

(A) Single cell suspensions from positive cell types were obtained by gentle trypsinization. Non-viable cells were excluded by 7AAD staining. Monoclonal antibodies specific for ABCB1, ABCB6, ABCG2, ABCC1 and ABCA1 were used to detect transporter expression as described in Materials and Methods. R1: cell population with marker positivity was gated based on the relevant isotype-matched control mAbs

 (B)The positive cell types were passaged into eight-well chambers for confocal microscopy. The samples were stained with specific antibodies to visualize ABCA1, ABCB1, ABCB6, ABCC1, ABCG2 and ABCC6 proteins (green) as described in the "Materials and methods"

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section. Nuclei were counterstained with Hoechst33342 (blue). Stained samples were examined by an Olympus FV500-IX confocal laser scanning microscope.

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A

B

C   Highest expression (5<Ct>15)

hES	Cardiac	Neural	MSC
FN1	AFP	PAX6	FN1
LIN28	ACTC	SFRP2	COL1A1
Nanog	GATA4	AFP	LAMB1
DNMT3B	COL1A1	SST	LAMC1
IFITM1	NPPA	FN1	IL6ST
PODXL	EBAF	EOMES-T	IMP2-IGFII

182x190mm (300 x 300 DPI)

hESC

239x149mm (300 x 300 DPI)

MSCl

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ABCB1	ABCB6	ABCG2	ABCC1	ABCA1

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253x171mm (300 x 300 DPI)

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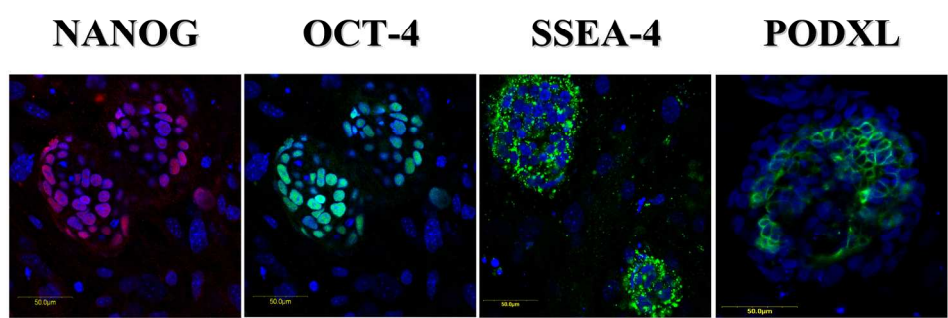41
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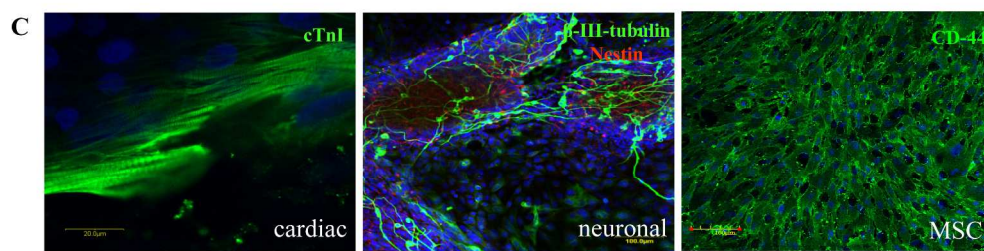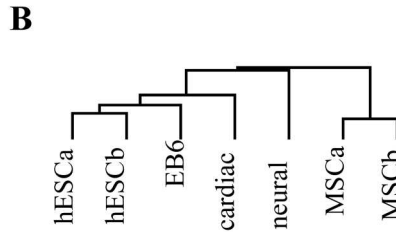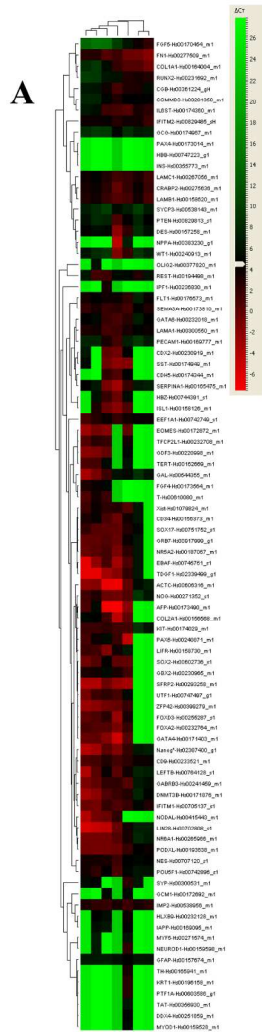47
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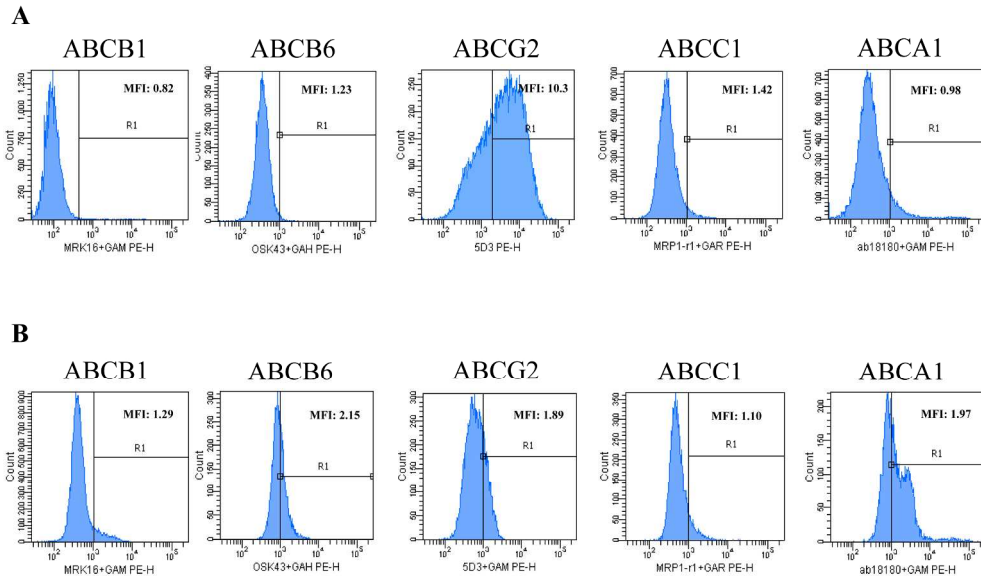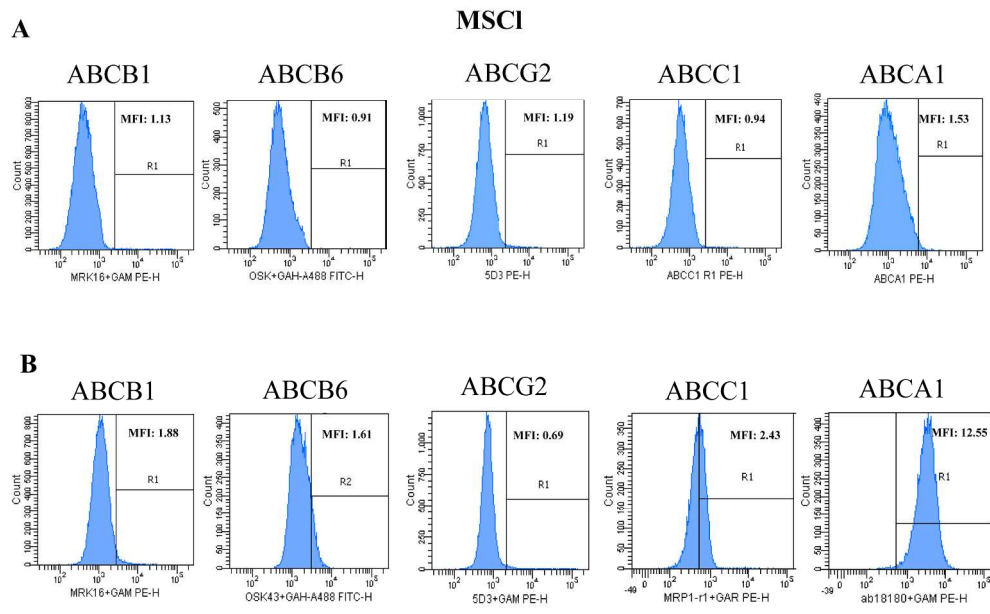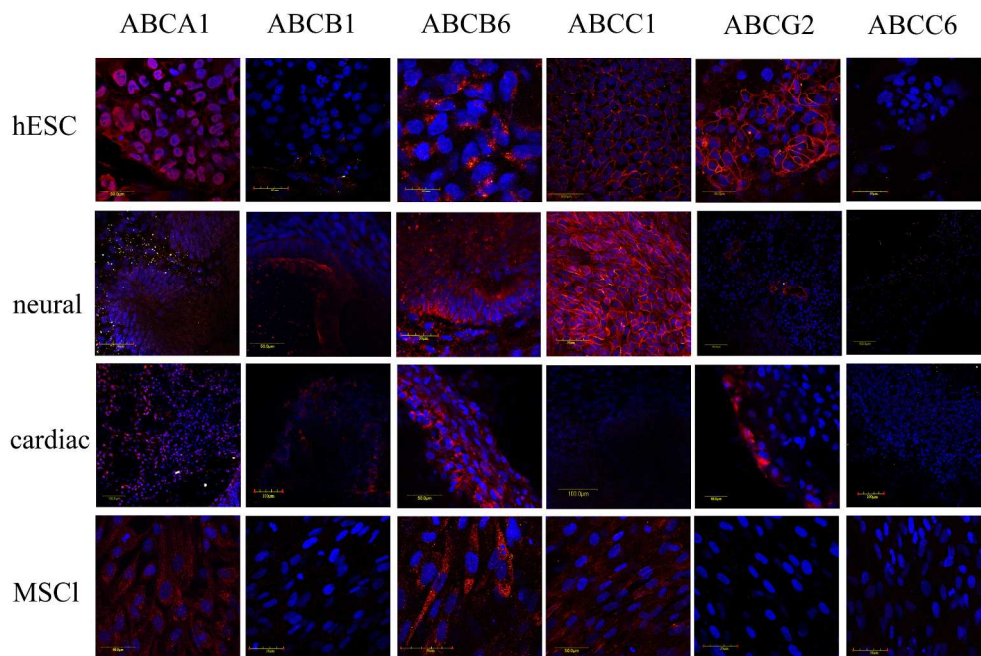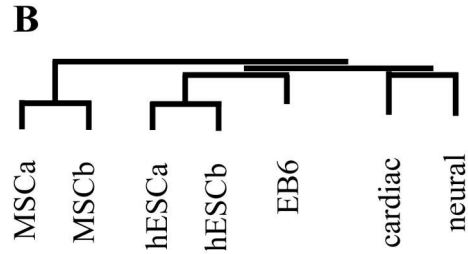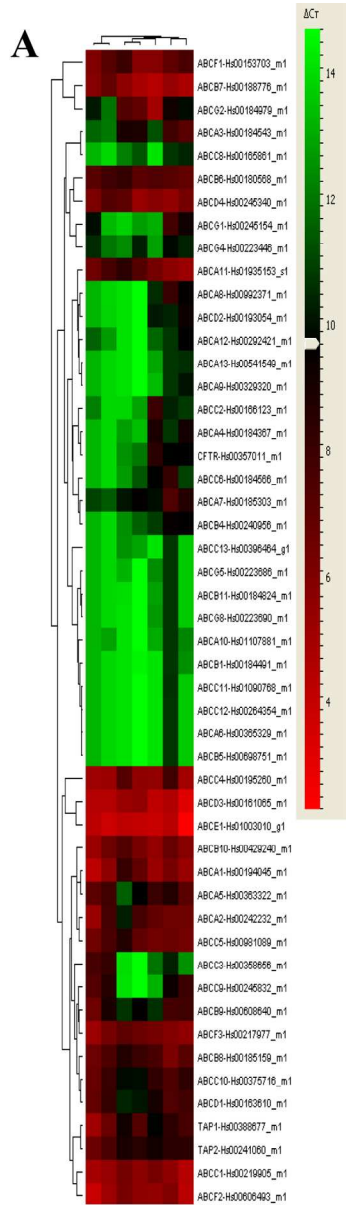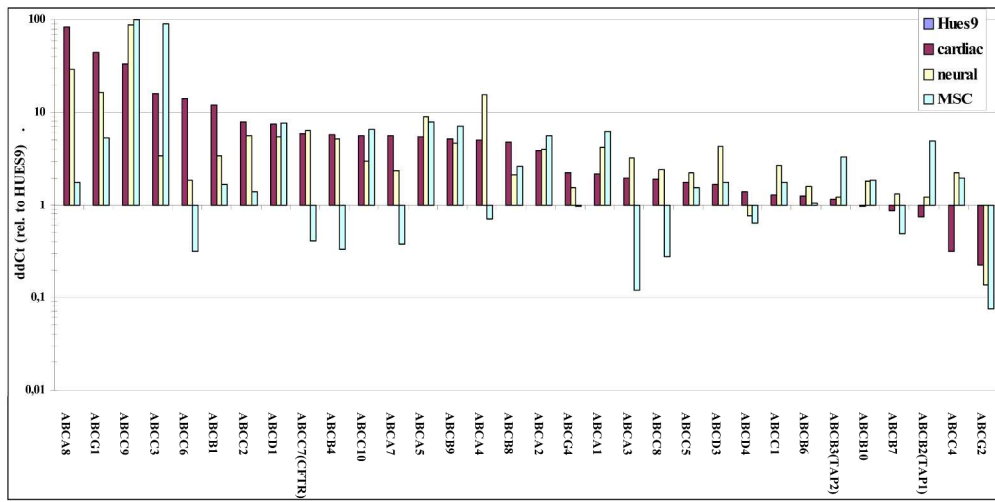253x129mm (300 x 300 DPI)

Suppl table 1

ABC protein (Gene symbol)	Function – potential substrates	Localization (protein)	Tissue distribution
ABCA1	HDL biogenesis –glycero-phospholipid compounds	PM/Golgi/lyso	Broad
ABCA2	Control of lipid transport - unknown	PM/Golgi/lyso	Brain- Broad
ABCA3	Lung surfactant production- glycero-phospholipid compounds	PM/Lysosome	Lung- Broad
ABCA4	Retinal integrity- glycero-phospholipid compounds	PM	Retina
ABCA5	Control of lipid transport - unknown	Endolysosome	Broad -Heart?
ABCA6	Control of lipid transport - unknown	PM	Broad
ABCA7	Phagocytosis (?)-glycero-phospholipid compounds/ceramide backbone lipids	PM	Broad
ABCA8	Control of lipid transport - unknown	PM	Broad
ABCA9	Control of lipid transport - unknown	PM	Broad
ABCA10	Control of lipid transport - unknown	PM	Broad
ABCA12	Skin lipid barrier formation- ceramide backbone lipids	PM	Keratinocytes/skin
ABCA13	Control of lipid transport - unknown	PM	Broad
ABCB1 (MDR1)	Multidrug resistance - drugs and metabolites	PM	Tissue barriers-Broad-cancer
ABCB2 (TAP1)	Antigen presentation - peptide	ER	Blood
ABCB3 (TAP2)	Antigen presentation - peptide	ER	Blood
ABCB4	Phospholipid flippase - lipids	PM	Liver canaliculus
ABCB5	Multidrug resisitance - drugs	PM	Broad-melanomas
ABCB6	Heme and porphyrin transport?	Mito/lyso/PM?	Broad
ABCB7	Metal homeostasis - heme	Mitochondria	Broad
ABCB8	Mitochondrial iron homeostasis - unknown	Mitochondria	Broad
ABCB9	Antigen presentation-peptide?	Lysosomal	Testis-broad
ABCB10	Mitochondrial transport functions related to heme biosynthesis?	Mitochondria	Broad
ABCC1 (MRP1)	Multidrug resistance-drugs/ metabolites/ organic anions	PM	Tissue barriers – broad - cancer
ABCC2	Multidrug resistance - drugs/ metabolites/ organic anions	PM	Broad-liver
ABCC3	Multidrug resistance - drugs/ metabolites/ organic anions	PM	Broad
ABCC4	Multidrug resistance - drugs/ metabolites/ organic anions	PM	Broad
ABCC5	Multidrug resistance - drugs/ metabolites/ organic anions	PM	Broad
ABCC6	Tissue metabolism metabolites/ organic anions	PM	liver, broad
ABCC7 (CFTR)	Cystic fibrosis - chloride ion channel	PM	Ephitelial cells
ABCC8 (SUR1)	Sulfonylurea receptor – potassium channel	PM	Pancreas
ABCC9 (SUR2)	Sulfonylurea receptor – potassium channel	PM	Smooth/cardiac muscle
ABCC10	Multidrug resistance - drugs/ metabolites/ organic anions	PM	Broad
ABCC11	Multidrug resistance - drugs/ metabolites/ organic anions	PM	Broad - liver
ABCC12	Multidrug resistance - drugs/ metabolites	PM	Broad
ABCD1	acyl–CoA ester transporter?	Peroxisome	Broad
ABCD2	Long chain fatty acids? - unknown	Peroxisome	Broad
ABCD3	Long chain fatty acids? - unknown	Peroxisome	Broad
ABCD4	Long chain fatty acids? - unknown	Peroxisome	Broad
ABCE1*	RNase L inhibitor	Cytoplasm	Broad
ABCF1*	Translation initiation/Inflammation	Cytoplasm	Broad
ABCF2*	Inflammation?	Cytoplasm	Broad
ABCF3*	Inflammation?	Cytoplasm	Broad
ABCG1	Lipid homeostasis	PM	Broad /macrophage
ABCG2	Multidrug resistance - drugs/ metabolites	PM	Tissue barriers-stem cells-cancer
ABCG5	Sterol transporter	PM	Liver/colon/intestine
ABCG8	Sterol transporter	PM	Liver/colon/intestine

no membrane domain, not a transporter; lyso-Lysosome, Mito-Mitochondria, PM- plasmamembrane
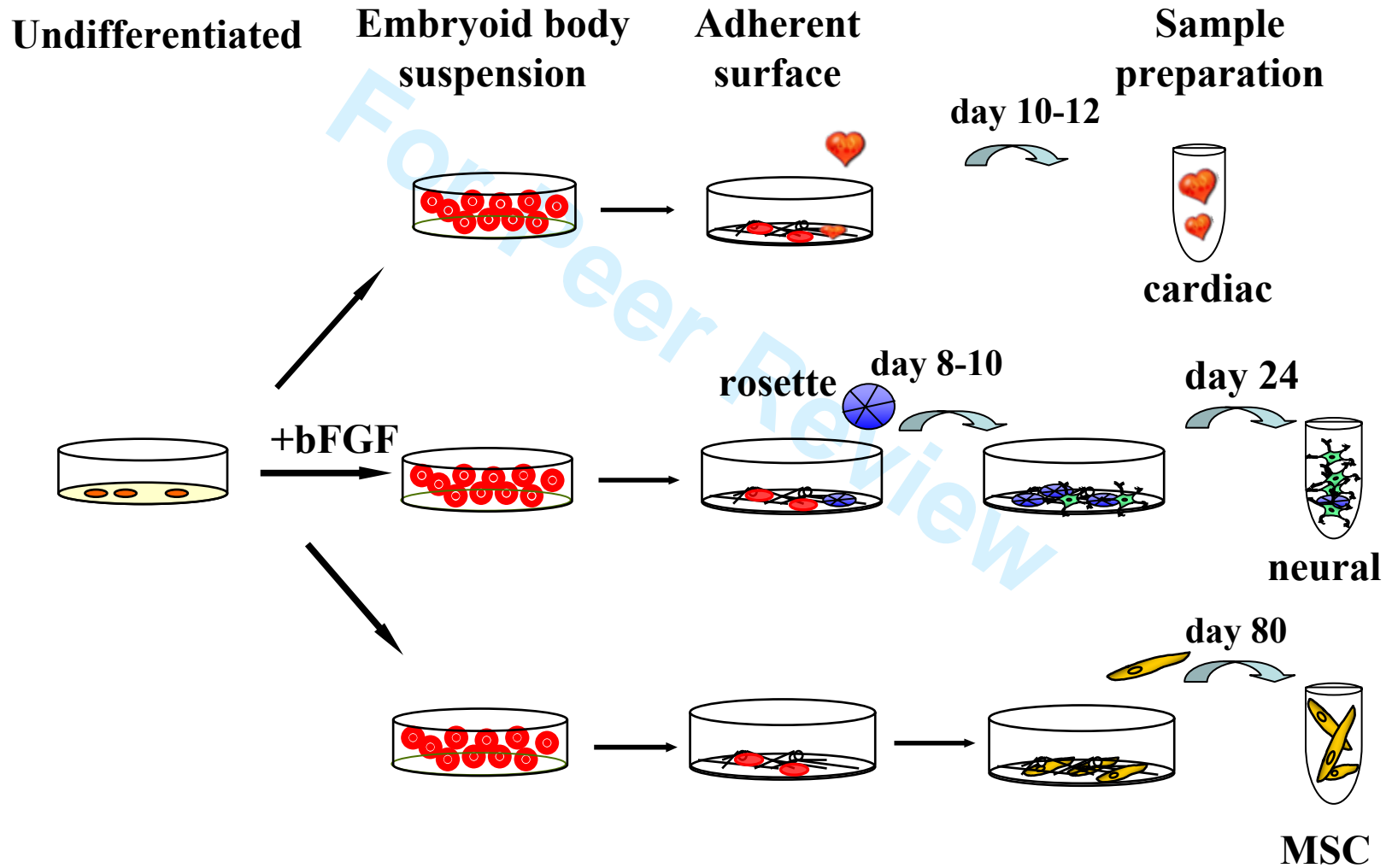
Suppl table 2

Assay	HUES9	HUES9-EB6	cardiac	neural	MSC
ACTC	9.02465	3.7571	7.4739	10.0412	17.9791
AFP	14.2674	3.2519	6.8672	8.0137	40
CD34	14.6264	11.385	13.7984	13.7884	28.5567
CD9	12.0632	13.1759	15.6704	12.4877	15.0844
CDH5	40	12.4599	15.0009	14.8382	40
CDX2	29.35035	10.2434	13.6817	14.8291	40
CGB	17.5176	14.0215	15.5874	13.1917	15.59355
COL1A1	20.04095	15.9393	16.3291	10.8368	11.05185
COL2A1	15.95785	11.9137	9.8554	9.9986	40
COMMD3	18.7591	15.2432	17.398	14.5128	15.49605
CRABP2	14.7185	12.873	14.1725	12.0959	14.092
DDX4	40	40	40	14.8892	40
DES	16.00475	15.3966	12.2715	12.3499	18.46895
DNMT3B	8.455	10.9973	16.1406	12.9043	17.5691
EBAF	6.7348	11.5204	11.1274	11.0091	28.5409
EEF1A1	13.0308	14.3224	16.3732	13.7074	15.2213
EOMES	9.0693	8.509	40	9.9145	40
FGF4	14.59295	15.4906	40	40	40
FGF5	26.62775	26.2507	25.5898	16.6505	14.09525
FLT1	15.0133	14.1613	18.1986	12.3948	17.09715
FN1	13.71435	10.8159	16.014	9.5729	9.8765
FOXA2	11.9286	10.8536	13.1473	11.9565	40
FOXD3	11.49785	12.7884	11.6814	13.4076	40
GABRB3	10.32995	12.5589	16.5645	10.6071	17.3419
GAL	8.7945	11.9739	21.4419	15.3335	18.80415
GATA4	10.89805	8.4833	9.1121	10.4641	40
GATA6	15.9316	12.2737	15.4269	12.7032	16.9483
GBX2	14.3226	15.8008	17.8192	11.5798	40
GCG	21.3889	21.0119	20.351	20.0283	18.2467
GCM1	40	15.8259	40	15.6714	40
GDF3	9.3102	10.1617	40	15.1628	40
GFAP	17.9208	17.4083	21.3562	14.7978	17.5945
GRB7	11.0001	11.0428	14.0085	14.1018	28.5735
HBB	40	40	40	40	40
HBZ	28.0218	12.2503	13.8863	15.622	40
HLXB9	27.2928	15.9442	40	13.9075	40
IAPP	27.9475	16.9931	40	14.3921	40
IFITM1	10.26225	12.3415	15.1743	11.9693	15.18425
IFITM2	14.81865	14.4135	17.0487	13.7372	13.7668
IL6ST	17.31295	11.9074	15.4612	10.7083	14.02905
IMP2	13.7687	12.274	17.5108	11.7457	14.0564
INS	40	40	40	40	40
IPF1	40	13.7921	40	40	40
ISL1	26.57485	9.9308	12.3948	10.8573	40
KIT	12.8713	12.7337	15.7758	11.3714	19.01075
KRT1	40	40	40	12.5333	40
LAMA1	14.3862	11.607	15.9882	13.3069	15.55865
LAMB1	14.3738	12.2547	15.0709	11.475	13.50285
LAMC1	15.35335	13.3624	15.8309	11.9612	13.5349
LEFTB	9.37445	14.3882	14.5416	13.7295	18.55825
LIFR	12.6536	8.7835	18.3789	16.9181	40
LIN28r	5.7509	5.9537	13.8717	13.6844	18.707
MYF5	28.5556	40	40	16.3116	40
MYOD1	40	40	40	15.461	40
Nanog	7.45745	10.6751	12.6507	12.0726	18.2995
NES	14.57705	13.5591	15.9564	14.6324	16.0901
NEUROD1	28.49865	40	40	12.2476	40
NODAL-	8.60645	9.9404	16.6394	40	40
NOG-	13.311	9.8815	13.5484	10.8616	19.1265
NPPA	40	40	9.9905	17.8166	40
NR5A2	11.02495	11.5895	13.138	12.4778	28.52985
NR6A1	10.09355	9.41	14.9261	13.5482	18.6665
OLIG2	28.02605	40	40	40	40
PAX4	40	40	40	40	40
PAX6	13.5282	15.7499	16.1567	5.3158	40
PECAM1	19.03115	13.6099	15.5921	17.3972	20.1134
PODXL	10.16825	9.4115	13.8094	12.126	15.40145
POU5F1	13.9814	14.8422	15.1845	14.9591	17.47575
PTEN	18.17615	18.0092	15.2378	14.3919	17.3416
PTF1A	40	40	40	12.5194	40
REST	14.9088	11.9599	19.73	15.1429	15.5926
RUNX2	20.91325	16.6927	20.4313	13.0368	14.33975
SEMA3A	13.80575	12.2443	16.8448	11.5796	15.aug
SERPINA1	16.59335	9.2811	11.2344	12.0315	18.48075
SFRP2	7.35165	9.8061	11.6903	6.7405	40
SOX17	12.35825	10.5468	14.3839	12.9334	27.95815
SOX2	10.9182	14.5192	14.2687	9.9534	40
SST	28.6381	9.6961	12.2637	8.4432	40
SYCP3	18.83445	21.562	20.9011	17.3241	20.1134
SYP	16.08965	40	40	12.4536	28.5875
TAT	40	40	40	16.6877	40
T-Brachyury	13.2558	11.942	40	40	40
TDGF1	4.5432	7.1901	12.6029	12.1461	28.6227
TERT	12.06875	15.7659	40	16.3876	40
TFCP2L1	12.53975	12.2961	40	13.5906	40
TH	40	40	40	13.4476	40
UTF1	11.3367	12.6754	15.5858	15.4084	40
WT1	18.48415	20.9425	12.7707	12.0399	20.1134
Xist	13.79355	12.7688	17.0112	10.6535	28.591
ZFP42	8.6635	9.5933	14.0637	12.089	40
18S	13.7056	14.4596	15.7815	16.4268	14.1812
ACTB	10.98405	10.1714	14.3886	9.4178	10.5703
BRIX	12.78025	13.2652	18.2174	13.2374	15.18775
CTNNB1	13.1781	12.0935	14.8335	11.6393	13.57145
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RAF1	15.10905	15.136	16.8423	13.2651	15.707
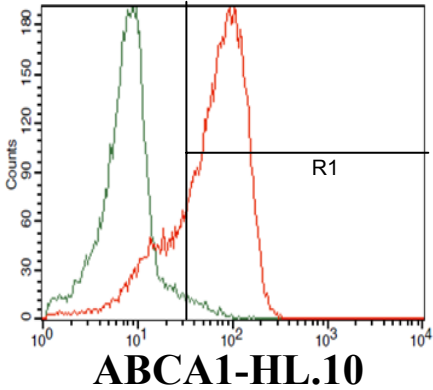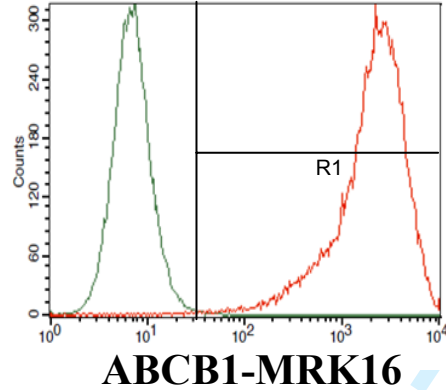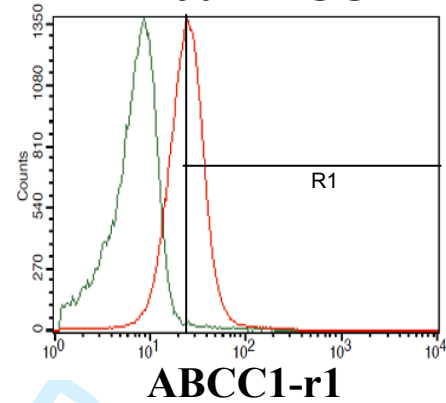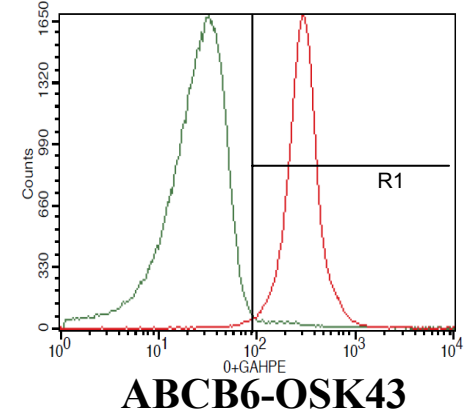
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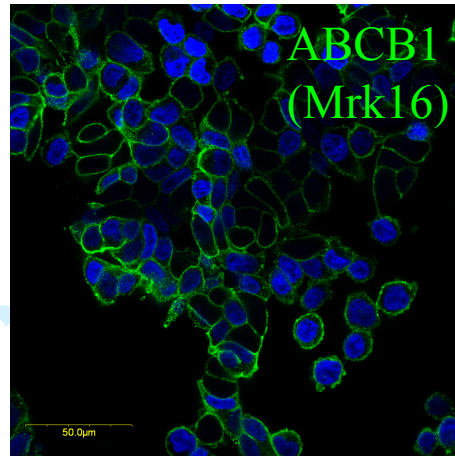
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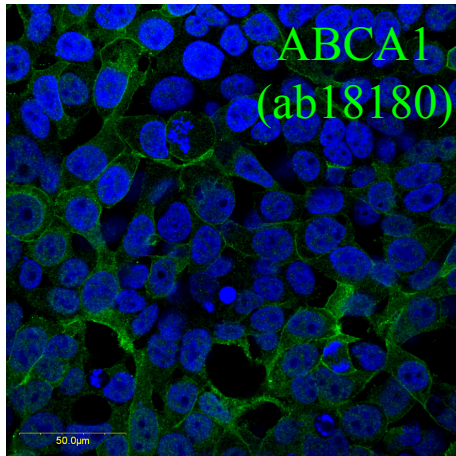
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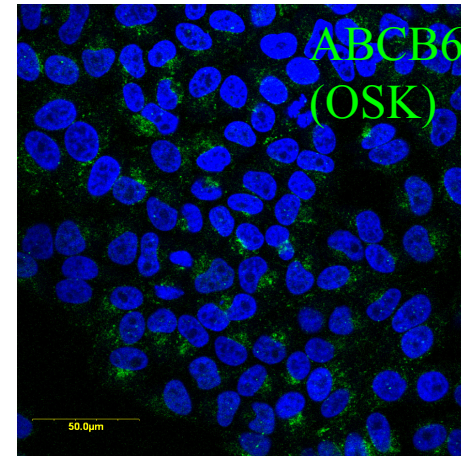
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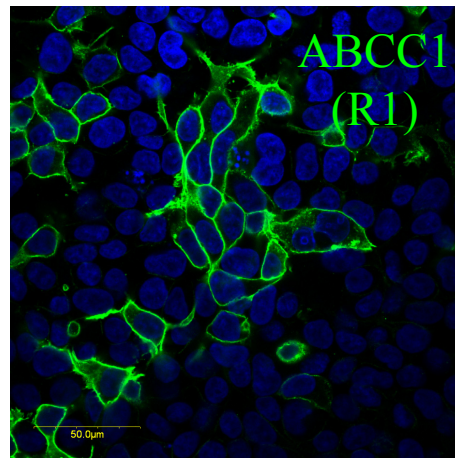
MDCK-C6
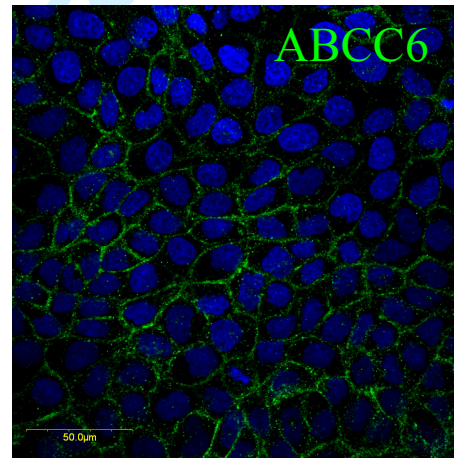
Suppl table 3

Assay	HUES9	HUES9-EB6	cardiac	neural	MSC
ABCA1	31.0396	28.9795	33.4435	29.5752	29.23925
ABCA10	40	37.5343	40	37.3005	38.8018
ABCA11	31.3768	30.068	32.8675	29.4832	31.4277
ABCA12	40	35.4942	40	34.0227	36.8993
ABCA13	40	37.4678	40	34.9098	40
ABCA2	32.55955	30.6405	33.7526	30.7606	30.81145
ABCA3	32.4187	35.0962	35.1046	31.4391	36.60345
ABCA4	37.36255	32.6502	40	33.397	40
ABCA5	34.1788	31.7675	35.9395	31.3826	31.76785
ABCA6	40	40	40	40	40
ABCA7	33.4291	33.7757	34.6049	32.8954	35.6226
ABCA8	40	34.3586	35.2636	33.8209	40
ABCA9	40	37.8515	40	34.3748	40
ABCB1	38.93785	40	39.9477	36.8391	40
ABCB10	30.30915	29.2057	33.9407	30.0782	30.2309
ABCB11	40	37.3311	40	40	40
ABCB2(TAP1)	31.81895	33.5447	35.6379	31.9229	30.23265
ABCB3(TAP2)	32.2998	32.9817	35.7159	32.6841	31.4224
ABCB4	35.6415	34.701	36.7285	33.911	40
ABCB5	40	40	40	40	40
ABCB6	31.17235	31.1244	34.3911	31.094	31.8473
ABCB7	28.77805	28.2054	32.6029	29.0442	30.66375
ABCB8	31.90185	31.3394	33.2875	31.4903	31.34925
ABCB9	33.7305	34.4125	34.9418	32.1476	32.058
ABCC1	29.50465	30.0096	32.7507	28.7592	29.4335
ABCC10	33.46015	32.0802	34.6533	32.6083	31.72
ABCC11	40	40	40	40	40
ABCC12	40	40	40	40	40
ABCC13	36.34535	40	40	40	40
ABCC2	37.2038	31.7999	38.2795	35.1388	38.7928
ABCC3	39.61925	35.7791	38.2363	37.5311	32.25395
ABCC4	29.94055	29.1956	35.0429	29.2441	29.50745
ABCC5	31.30305	30.1312	33.886	30.6196	31.31055
ABCC6	35.98755	33.5331	35.3876	35.9696	40
ABCC7(CFTR)	35.9023	32.2108	37.0983	33.9348	40
ABCC8	35.3508	40	40	34.7305	40
ABCC9	40	37.6908	36.5667	32.2507	32.30205
ABCD1	33.817	32.8194	34.5989	32.0951	31.8303
ABCD2	40	33.9739	38.0008	33.6023	39.4229
ABCD3	28.8863	27.9738	31.7988	27.4914	28.8246
ABCD4	29.6969	29.5948	32.6149	30.5656	31.02785
ABCE1	27.68005	27.6752	31.5605	26.5991	28.ápr
ABCF1	30.43265	29.5735	34.1468	31.8244	30.94155
ABCF2	29.4323	29.237	33.205	28.58	28.84575
ABCF3	30.36395	30.0931	33.1239	29.77	30.05705
ABCG1	37.55575	37.6022	35.0021	33.4688	36.36145
ABCG2	30.6401	28.7107	37.1048	34.228	35.7986
ABCG4	34.9418	29.6009	37.0756	34.6503	35.6558
ABCG5	38.887	36.2582	40	40	40
ABCG8	40	37.1797	40	40	40
18S	14.1835	14.1306	16.2589	15.2277	14.23325
ACTB	21.99195	21.7459	26.1247	21.3886	21.46675
GAPDH	23.2931	22.6533	26.8245	23.6486	23.33645
GUSB	29.8687	28.8575	33.3999	30.6198	30.85525
HMBS	29.96835	30.5808	33.7787	31.3743	31.0917
HPRT1	26.02505	26.2342	31.099	27.2207	27.99935
PGK1	24.86525	24.9142	27.0533	24.7633	26.20165
POLR2A	29.03875	28.0373	32.8166	28.4542	29.20705
PPIA	23.60035	23.8241	27.2754	24.3327	24.40375
RPLP0	24.1812	24.0738	26.5179	24.1969	24.5465
TBP	29.30135	28.6037	33.2622	29.4393	29.942
YWHAZ	28.95445	28.18	32.5673	29.0133	31.8896

10-100x higher expression relative to hESC

Cardiac		Neural		MSC	
ABCA8	83.6	ABCC9	87.8	ABCC9	99.2
ABCG1	44.7	ABCA8	29.6	ABCC3	90.2
ABCC9	33.9	ABCG1	16.8		
ABCC3	16.3	ABCA4	16.0		
ABCC6	13.8				
ABCB1	11.9				

2-10x higher expression relative to hESC

Cardiac		Neural		MSC	
ABCC2	7.77	ABCA5	9.04	ABCA5	7.89
ABCD1	7.39	ABCC7	6.40	ABCD1	7.61
ABCC7	5.98	ABCC2	5.62	ABCB9	7.10
ABCB4	5.71	ABCD1	5.45	ABCC10	6.48
ABCA7	5.58	ABCB4	5.23	ABCA1	6.17
ABCC10	5.58	ABCB9	4.65	ABCB2	4.96
ABCA5	5.48	ABCD3	4.35	ABCA2	5.62
ABCB9	5.16	ABCA1	4.21	ABCG1	5.37
ABCA4	5.06	ABCA2	4.00	ABCB3	3.35
ABCA2	3.86	ABCB1	3.47	ABCB8	2.62
ABCB8	4.76	ABCC3	3.45	ABCC4	2.01
ABCG4	2.27	ABCA3	3.28		
ABCA1	2.22	ABCC10	3.00		
		ABCC1	2.70		
		ABCC8	2.45		
		ABCA7	2.37		
		ABCC4	2.29		
		ABCC5	2.25		
		ABCB8	2.15		

<2 or lower expression relative to hESC

Cardiac		Neural		MSC	
ABCA3	1.98	ABCC6	1.91	ABCB10	1.89
ABCC8	1.95	ABCB10	1.82	ABCD3	1.82
ABCC5	1.79	ABCB6	1.63	ABCC1	1.80
ABCD3	1.69	ABCG4	1.59	ABCA8	1.78
ABCD4	1.43	ABCB7	1.33	ABCB1	1.70
ABCC1	1.31	ABCB2	1.26	ABCC5	1.57
ABCB6	1.27	ABCB3	1.26	ABCC2	1.41
ABCB3	1.19	ABCD4	0.77	ABCB6	1.05
ABCB10	0.96	ABCG2	0.14	ABCG4	0.95
ABCB7	0.87			ABCA4	0.71
ABCB2	0.74			ABCD4	0.63
ABCC4	0.32			ABCB7	0.48
ABCG2	0.23			ABCC7	0.41
				ABCA7	0.38
				ABCB4	0.33
				ABCC6	0.32
				ABCC8	0.27
				ABCA3	0.12
				ABCG2	0.07

Supplementary methods

Cell cultures and differentiation

ABCA1 overexpressing HEK 293 (HEK 293-A1), ABCC1 overexpressing HEK 293 (HEK 293-A1), ABCC6 overexpressing MDCK (MDCK-C6), and HeLa (endogenously expressing ABCB6) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal calf serum, 1% of L-Glutamine, and 1% of penicillin/streptomycin (Life Technologies) and were used as positive controls for immunostainings of respective ABC transporter proteins. HCT-15, ABCA1, ABCB1 and ABCC1 overexpressing HL60 cell lines (HL60-ABCA1, HL60-ABCB1 and HL60-ABCC1 respectively) were cultured in RPMI1640 Medium, supplemented with 10% of fetal calf serum, 1% of L-Glutamine, and 1% of penicillin/streptomycin (Life Technologies) and was used as a positive control cell line for respective ABC transporter proteins stainings.

HUES9 cells were cultured on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (mitomycin-C (Sigma) and Millipore – CF1) on tissue culture 6- well plates (Nunc). The culture medium consisted of 15% Knockout Serum Replacement (Gibco), 80% Knockout Dulbecco Modified Eagle Medium (Gibco), 1 mM Glutamax-I (Gibco), 0.1 mM beta-mercaptoethanol, 1% nonessential amino acids, and 4 ng/mL human fibroblast growth factor (Invitrogen). Cells were re-plated every second day on fresh feeder layer by enzymatic dissociation with 0,025% trypsin-EDTA (Invitrogen).

Differentiation of Human Embryonic Stem Cells (HUES9)

Spontaneous differentiation of the HUES9 cells was performed via embryoid body (EB) formation system in suspension. Undifferentiated cells were passaged enzymatically with collagenase-IV and were transferred to Poly-2 Hydroxyethyl-methacrylate (Sigma-Aldrich) treated Petri dishes; therefore the spontaneous aggregation of cells and embryoid body formation could be obtained. EBs were cultured in differentiation medium consisting of Ko DMEM supplemented with 20% non heat inactivated fetal bovine serum, 1% nonessential amino acids, 1mML-glutamine, and 0.1mM beta-mercaptoethanol (Invitrogen) for six days, medium was changed daily. In case of neuronal differentiation, similarly to undifferentiated cell culture medium, 4 ng/ml bFGF was added to the culture media during EB formation. After 6 days, the embryoid bodies were placed onto gelatin-coated 24- well plates, where they continued a further spontaneous differentiation process in D-MEM supplemented with 10% of fetal calf serum, 1% of L-Glutamine, medium was changed on every other day. After few

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days of culturing under the aforementioned conditions several cell types were formed in the gelatin-coated culture dishes, including cardiomyocytes, neuronal progenitors and fibroblast-like cells. These cells were identified by morphological signs under phase contrast light microscope and mechanically passaged for further propagation in D-MEM supplemented with 10% of fetal calf serum, 1% of L-Glutamine.

Flow cytometry and immuno –cytochemical labeling

For flow cytometric analysis single cell suspensions were split into two portions. First portions of cells remained intact and were labeled in PBS containing 0.5% bovine serum albumin with appropriate antibodies. The second samples were fixed and permeabilized by the Fix&Perm (Invitrogen) solution according to the manufacturer's instruction before staining procedure. The labeling was accomplished with specific antibodies listed below in the table. In all hESC samples an anti-mouse Sca-1 (Ly-6A/E) (FITC or PE, BD Pharmingen) antibody was employed for gating out the positively labeled mouse feeder cells. Non-viable cells were excluded by 7AAD staining. Samples were analyzed by a BD FACSCantoII flow cytometer (Becton Dickinson Immunocytometry Systems [BDIS]), equipped with a 488 nm diode laser (emission filters: 585/42 nm for PE; 530/30 for FITC, 670LP for 7AAD) and a 633 nm He-Ne laser (emission filters: 660/20 for APC) with BD FACSDiva 6.1.2 Software (BDIS).

For confocal microscopy the cells were seeded onto eight-well Nunc Lab-Tek II Chambered Coverglass (Nalge Nunc International), and fixed with 4% paraformaldehyde in Dulbecco's modified PBS (DPBS) or Fix&Perm A solution for 15 min at room temperature. Occasionally 5 min methanol treatment was used (see the Table). Following further washing steps with DPBS, nonspecific antibody binding was blocked for 1h at room temperature in blocking solution (DPBS containing 2mg/ml bovine serum albumin,1% fish gelatin, 5% goat serum) and with (Complete) or without 0.1% Triton-X 100 (Complete wo Tx) or Fix&Perm B solution. The cells were then incubated for 1 h at room temperature with specific monoclonal antibodies in proper blocking solutions. After washing with DPBS, the samples were incubated for 1 h at room temperature with secondary antibodies. After removing secondary antibody solutions and washing steps Hoechst33342 (Invitrogen) was used for nuclear staining.

Table of antibodies:

Primary antibody	Manufacturer	Dilution	Secondary antibody	Labeling methods
FACS				
Sca-1 (Ly-6A/E) FITC or PE	BD Pharmingen	1:25	-	Intact
SSEA4-APC	R&D Systems	1:50	-	Intact
PODXL-PE	R&D Systems	1:10	-	Intact
Anti ABCG2 5D3-CD338	BD Pharmingen	1:125	Mouse IgG2b-PE	Intact+Ko143 or Fix&Perm+Ko143
Anti ABCB1 MRK16	Alexis Biochemicals	1:100	Mouse IgG2a-PE	Intact or Fix&Perm
Anti-Lan clone: OSK43	Provided by H. Takahashi	1:70	IgG+M-A488 or PE	Intact or Fix&Perm
Anti ABCC1 clone: MRPr1	Abcam	1:25	Rat IgG2a-PE	Intact or Fix&Perm
Anti-ABCA1 clone:AB.H10	Abcam	1:50	Mouse IgG1-PE	Intact or Fix&Perm
Immuncytochemistry				
Anti ABCG2 5D3-CD338	BD. Pharmingen	1:250	Mouse IgG2b-PE	Fix&Perm +Ko143
Anti ABCB1 MRK16	Alexis Biochemicals	1:100	Mouse IgG2a-PE	4%PFA Complete wo Tx
Anti-Lan clone: OSK43	Provided by H. Takahashi	1:100	IgG+M-A488 or PE	Fix&Perm
Anti ABCC1 clone: MRPr1	Abcam	1:25	Rat IgG2a-PE	4%PFA+ 5 min methanol Complete
Anti-ABCA1 clone:AB.H10	Abcam	1:200	Mouse IgG1-PE	Fix&Perm
Anti ABCC6 M6II-7	Pierce Antibodies	1:100	Rat IgG2a-A488	4%PFA +5 min methanol Complete
Oct4	SantaCruz	1:50	Mouse IgG-A488	4%PFA Complete
Nanog	RnD Systems	1:10	Goat-cy3	4%PFA Complete
SSEA4	RnD Systems	1:50	Mouse IgG-A488	4%PFA Complete wo Tx
PODXL	RnD Systems	1:10	Mouse IgG-A488	4%PFA Complete wo Tx
cardiac Troponin-I	Sigma	1:300	Mouse IgG-A568	4%PFA Complete
β-III Tubulin	RnD Systems	1:2000	Mouse IgG-A488	4%PFA Complete
CD-44-FITC	BD Pharmigen	1:10	-	4%PFA Complete wo Tx
Nestin	Abcam	1:250	Rabbit IgG-A564	4%PFA Complete

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