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Review

Inconsistency in using early differentiation markers of human pluripotent stem cells

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Advances in the field of human pluripotent stem cells (hPSC) have prompted researchers to advocate for the increased development of dependable therapies to cure degenerative diseases and replace damaged tissues. hPSCs have a one-of-a-kind ability to differentiate into all cell types in the body. The ability to characterise homogeneous primary cell populations, such as pluripotent stem cells and germ layer cells, is required for the efficient generation of adult cells. Several in vitro differentiation protocols for germ layer lineages have been extensively researched. There is, however, no standard set of markers that can be used to separate endoderm, ectoderm, and mesoderm populations from hPSC differentiation cultures. This review discusses the inconsistency among studies in identifying endodermal, mesodermal, and ectodermal cells using markers. The search was restricted to markers used in the last 5 years to identify differentiated cells of the three germ layers from hPSCs. The focus of this review, however, is on the most commonly used early differentiation markers.

Keywords: Differentiation markers, ectoderm markers, mesoderm markers, endoderm markers, Early differentiation.

INTRODUCTION

Human pluripotent stem cells (hPSCs) are unspecialized cells that can undergo indefinite self-renewal and differentiate into all cell types of a human body, excluding extra-embryonic tissues. The main types of hPSCs include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Romito and Cobellis, 2016). The remarkable capacity of hPSCs to differentiate into all the cell lineages renders them an attractive method for replacing damaged tissues, testing the toxicity of drugs and studying the mechanisms of diseases (Dakhore et al., 2018). During the earliest developmental stages of the human embryo, an important process known as gastrulation takes place (Figure 1). Gastrulation involves the formation of three germ layers (ectoderm, endoderm and mesoderm) from pluripotent epiblast cells. Each layer, which is no longer pluripotent, will later give rise to specific body organs and tissues (Muhr and Ackerman, 2020).

In vitro generation of any differentiated cell type from hPSCs is preceded by the formation of ectoderm, mesoderm or endoderm cells. Detecting the expression of cell type-specific markers is a critical tool for confirming the differentiation status of hPSCs (Liu and Zheng, 2019). Therefore, it is essential to validate the quality of germ layer differentiation to generate efficient adult cells, such as functional hepatocytes (Zakrzewski et al., 2019). In the last 5 years, a wide range of markers have been used in several studies to identify induced early ectoderm, endoderm or mesoderm cells from hPSCs (Tables 1, 2 and 3). However, there are inconsistencies between these studies due to the lack of cross-study validation protocols. This review describes the inconsistency among studies in using the markers for identifying endodermal, mesodermal, and ectodermal cells. Figure 2 represents the detailed methodology used to select the early differentiation markers of hPSCs that have been used in the last 5 years in the literature. However, the focus of this review is directed

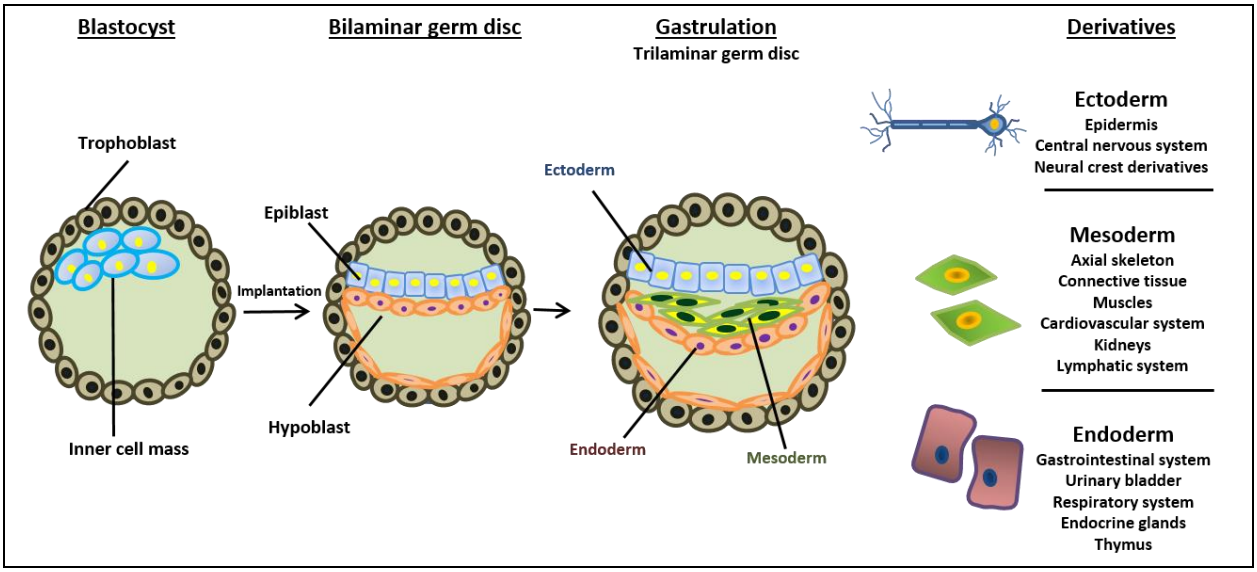

Figure 1: Schematic diagram showing the process of gastrulation. After implantation, the inner cell mass differentiates to form the bilaminar germ disc, which consists of the epiblast and the hypoblast. The bilaminar germ disk then differentiates further into a trilaminar embryo consisting of the three distinct germ layers: ectoderm, mesoderm, and endoderm. Cells of each germ layer eventually give rise to specific tissue types in the human body.

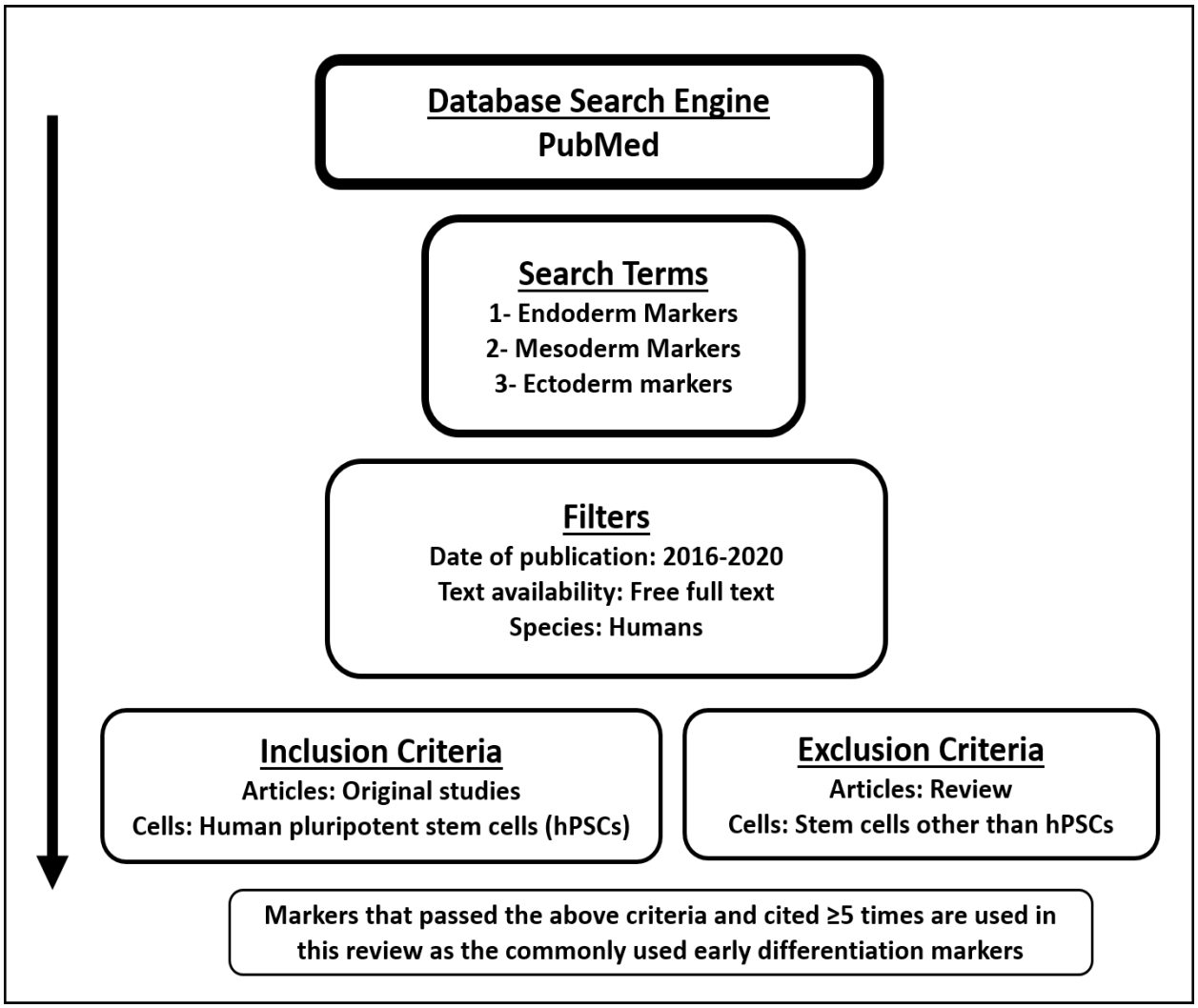

Figure 2. Methodology used to select the early differentiation markers of human pluripotent stem cells.

Table 1. The ectoderm markers cited in scientific literature in the last 5 years

Marker	Official gene name
CDH1	Cadherin 1 (E-cadherin)
DCX	Doublecortin
FGF5	Fibroblast growth factor 5
IGF1	Insulin like growth factor 1
NCAM1	Neural cell adhesion molecule 1
NES	Nestin
NEUROD1	Neuronal differentiation 1
OTX1	Orthodenticle homeobox 1
OTX2	Orthodenticle homeobox 2
PAX6	Paired box 6
SOX1	SRY-box transcription factor 1
TUBB3	Tubulin beta 3 class III
ZIP2	Zinc finger E-box binding homeobox 2

to the most commonly used markers.

Ectoderm Markers

Ectoderm is the outermost layer of the three germ layers, which goes on to form the central and peripheral nervous systems (CNS and PNS respectively), the epidermis of the skin and neural crest derivatives (Kiecker et al., 2016).

Early in embryogenesis, the anterior part of the epiblast differentiates to form the ectoderm germ layer. Of note is the fact that the primitive streak does not traverse this region, unlike epiblast cells that form mesoderm and endoderm. Therefore, the culture medium used for the induction of ectoderm is devoid of serum and any primitive streak inducers; hence, the process is known as the default pathway. Inhibitors of ectoderm formation in media include the Activin/Nodal, bone morphogenetic protein (BMP) and WNT molecules (Leung et al., 2013; Liu et al., 2018; Williams et al., 2012). These signaling molecules are switched off during ectoderm induction in the developing embryo. The differentiating pluripotent stem cells produce endogenous fibroblast growth factors (FGFs) on which the cells depend to induce neuroectoderm (Zheng et al., 2010). However, when ectoderm is formed, the activation of the BMP-signaling pathway promotes epidermal derivatives, whereas it blocks neural subtype specifications (Bertero et al., 2015, Feng et al., 2014). Several markers have been used to identify induced ectoderm cells from hPSCs in the last 5 years. However, PAX6, NES, SOX1 and TUBB3, which are discussed below, have been the most commonly used markers for ectoderm differentiation (Table 1).

Paired Box Protein 6 (PAX6)

PAX6, also called oculorhombin, is a highly conserved transcription factor which in humans is encoded by the *PAX6* gene (Thakurela et al., 2016). PAX6 is a pivotal differentiation marker for the ectoderm germ layer lineage (Cvekl and Callaerts, 2017), and has been used as an ectoderm marker in several studies (Table 1). A recent study characterized different cell types with commonly used pluripotent and lineage specific markers. Among these markers, only PAX6 was shown to be reliably used as an ectoderm differentiation marker (Kuang et al., 2019).

Nestin (NES)

NES is an essential intermediate filament protein, which is mainly expressed by neural progenitor cells in the mammalian CNS (Neradil and Veselska, 2015). NES is among the most commonly used ectoderm differentiation markers (Table 1). Before neurogenesis, NES is expressed in almost all ectodermal neuroepithelial cells (Neradil and Veselska, 2015). NES, however, is not exclusively expressed in neuroectodermal cells. A recent study found a similarity in the level of NES expression between iPSCs and differentiated ectoderm cells. Accordingly, NES was regarded as an unreliable early ectoderm differentiation marker (Kuang et al., 2019).

SRY-box Transcription Factor 1 (SOX1)

SOX1 is a member of the sex-determining region Y-box B1 (SOXB1) subfamily (Feng et al., 2014). SOX1 has been used as an early marker of ectoderm cells in several studies (Table 1). The *in vivo* expression of SOX1 is associated with the proliferation of neuroepithelial cells, whereas the exit of neural cells from mitosis correlates with the subsequent downregulation of SOX1 (Imai et al., 2017). A strong correlation between the level of SOX1 expression and neural linage specification has also been demonstrated in human iPSCs. Researchers successfully isolated cells with a neural phenotype after inhibiting BMP and observed a significant increase in SOX1 levels (Zhang et al., 2018).

Cytoskeletal Tubulin Beta 3 Class III Protein (TUBB3)

TUBB3 is widely used as a marker for early neuroectoderm differentiation (Table 1). TUBB3 expression, however, is not limited to ectoderm and neuronal cells. It has been reported that TUBB3 is highly expressed in the developing neural crest cells-derived melanocytes (Sebastian et al., 2017). Based on transcriptomic analyses, researchers found no significant increase of TUBB3 transcripts in ectoderm

Table 2. The endoderm markers cited in scientific literature in the last 5 years

Marker	Official gene name
CXCR4	C-X-C motif chemokine receptor 4
CDX2	Caudal type homeobox 2
ECD	Ecdysoneless cell cycle regulator
EOMES	Eomesodermin
FOXA2	Forkhead box A2
GATA4	GATA binding protein 4
GATA6	GATA binding protein 6
GSC	Goosecoid homeobox
HNF1B	HNF1 homeobox B
KIT	KIT proto-oncogene, receptor tyrosine kinase
MIXL1	Mix paired-like homeobox
SOX17	SRY-box transcription factor 17
SOX7	SRY-box transcription factor 7
AFP	Alpha fetoprotein

differentiated cells compared to pluripotent stem cells (Daily et al., 2017; Kuang et al., 2019). Accordingly, it was recommended that TUBB3 might not be a reliable ectoderm marker in stem cell trilineage validation studies (Kuang et al., 2019). Therefore, careful assessment of TUBB3 expression as an ectoderm marker or a neuronal marker should be considered.

Endoderm Markers

Endoderm is the mass of cells that is located internally within ectoderm and mesoderm germ layers. Definitive endoderm differentiates into multiple organs during embryo development, including urinary, respiratory and gastrointestinal systems, along with many glands in the endocrine system (Kiecker et al., 2016).

The efficiency of endoderm induction from hPSCs is monitored through changes in gene expression patterns and/or expression of cell surface markers. Induction of efficient definitive endoderm lineage is the first step for efficient differentiation into functional endoderm derivatives (Holtzinger et al., 2015). In culture, endoderm cell derivation from human embryonic stem cells (hESCs) depends on Activin signaling (Wang et al., 2015). Translating these findings from the developing embryo has allowed researchers to differentiate Activin-induced endoderm cells to respiratory- and digestive-related organs such as the liver, lungs, stomach and pancreas (Luo et al., 2017, Yiangou et al., 2018). SOX17, FOXA2, CXRC4 and GATA4 have been the most commonly used endoderm markers (Table 2).

SRY-box Transcription Factor 17 (SOX17)

SOX17 is a pioneer marker of generated definitive endoderm cells from a variety of hPSCs (Table 2). The expression of SOX17 is critical for inducing stable definitive endoderm cells from hESCs (Irie et al., 2015). Expression of SOX17 has been detected in hESCs differentiating toward endodermal fates following the treatment of the cells with Activin A (Luo et al., 2017). Mutations in *SOX17* are associated with abnormal endoderm formation (Ogaki et al., 2016).

Forkhead Box A2 (FOXA2)

The DNA-binding protein FOXA2 belongs to the forkhead box superfamily (Li et al., 2017). FOXA2 is highly expressed in the endoderm cells of the developing embryo (Gosalia et al., 2015). Several studies have used FOXA2 as an endoderm marker during the *in vitro* differentiation of hESCs (Table 2). Besides, FOXA2 is necessary for the formation of numerous human tissues of endoderm origin. For instance, the role of FOXA2 during human pancreas development has been established, where FOXA2 knockout pluripotent stem cells failed to differentiate to pancreatic cells (Lee et al., 2019). Moreover, a recent study uncovered a novel role of FOXA2 at the early stages of embryogenesis. The researchers found that FOXA2, hepatocyte nuclear factor 4 alpha (HNF4A) and E1A binding protein p300 (EP300) are the three most important genes for the first division of the fertilized egg (Godini and Fallahi, 2019). In adults, FOXA2 has been detected in tissues derived from endoderm (liver) (Warren et al., 2020) and mesoderm (uterus) (Kelleher et al., 2017). Since FOXA2 is expressed early in the dividing zygote and also in non-endodermal derived tissues, the reliability of FOXA2 as an early endoderm marker needs more attention.

C-X-C Motif Chemokine Receptor 4 (CXCR4)

CXCR4 is another marker commonly used for assessing the efficiency of endoderm induction from hPSCs (Table 1). Multiple studies depend on CXCR4 expression to identify proper endoderm populations, whereas additional studies evaluate endoderm induction by co-expression of CXCR4 with other markers, mainly CD117 and epithelial cell adhesion molecule (EPCAM) (Diekmann et al., 2019, Holtzinger et al., 2015, Zhong et al., 2017). A study that identified HDE1 as an endoderm-specific antibody indicated that enrichment of definitive endoderm from mixed-lineage populations could not be obtained by only

Table 3. The mesoderm markers cited in scientific literature in the last 5 years

Markers	Official gene name
BMP4	Bone morphogenetic protein 4
BRA	Brachyury
CDX2	Caudal type homeobox 2
DCN	Decorin
DES	Desmin
EOMES	Eomesodermin
GATA2	GATA binding protein 2
GATA4	GATA binding protein 4
GSC	Goosecoid homeobox
HAND1	Heart and neural crest derivatives expressed 1
IGF2	Insulin like growth factor 2
KDR	Kinase insert domain receptor
MESP1	Mesoderm posterior bHLH transcription factor 1
MIXL1	Mix paired-like homeobox
MSX1	Msh homeobox 1
NCAM1	Neural cell adhesion molecule 1
NODAL	Nodal growth differentiation factor
PDGFB	Platelet derived growth factor subunit B
PDGFRA	Platelet derived growth factor receptor alpha
SMN1	Survival of motor neuron 1
TBXT	T-box transcription factor T
TNNT2	Troponin T2, cardiac type
TWIST1	Twist family bHLH transcription factor 1
WT1	WT1 transcription factor

using CXCR4. This indication was based on the analysis, which revealed higher levels of non-endodermal genes, such as POU class 5 homeobox 1 (*OCT4*) (pluripotency), Mix paired-like homeobox (*MIXL1*) (primitive streak) and *CD56* (mesoderm) in the HDE1^{-}CXCR4^{+} cells than in the HDE1^{+}CXCR4^{+}cells (Holtzinger et al., 2015). Taken together, lack of validation protocols results in such inconsistency upon using CXCR4 as an early endoderm marker.

GATA4

GATA binding protein 4 (GAT4) is a zinc-finger transcription factor that binds to the DNA sequence "*GATA*" (Yuan et al., 2014). GATA4 first appears, as a pioneer factor, during the embryonic stage of development in the endoderm layer (Fisher et al., 2017, Tiyaboonchai et al., 2017). Therefore, it has been used as an early endoderm marker in multiple studies (Table 2). The differentiation of hPSCs has provided evidence that GATA6 regulates GATA4 during the generation of the definitive endoderm (Fisher et al., 2017). GATA4 is crucial for the development and function of several endoderm-derived tissues, although, its expression has also been identified in tissues derived from the mesoderm, such as the heart (Tiyaboonchai et al., 2017).

Mesoderm Markers

Mesoderm is the middle germ layer between ectoderm and endoderm and forms diverse tissues, including the skeletal and muscular systems, kidney, cartilages and blood vessels (Kiecker et al., 2016). BMP4 activates FGF and TGFB/Activin/Nodal pathways; the inhibition of these signaling cascades results in repression of the BMP4 function to induce mesoderm (Gordeeva, 2019). Manipulation of different pathways can induce subpopulations derived from mesoderm. For example, cardiac mesoderm can be efficiently obtained from hESCs when Activin is added with BMP4 (Sa et al., 2014). BRA, KDR and MIXL1 are widely used as early markers of mesoderm induction (Table 3).

Brachyury (BRA)

The transcription factor BRA is an early marker of gastrulation and lineage specification in humans (Faial et al., 2015). BRA is the key maker of the primitive streak and is highly expressed in embryos as the mesoderm layer is formed (Faial et al., 2015, Zhou et al., 2018). Therefore, BRA is widely used as marker to identify mesoderm cells derived from pluripotent cells (Table 3). In cancer research, BRA has been implicated in epithelial-mesenchymal transition (EMT) and tumor progression to metastasis; therefore, it has been proposed as a candidate for human cancer immunotherapy (Hamilton et al., 2017).

Kinase Insert Domain Receptor (KDR)

KDR, also known as VEGFR-2, is an endothelial cell growth factor receptor tyrosine kinase. KDR plays a significant role in early vascular development and the regulation of vascular permeability (Modi and Kulkarni, 2019). KDR expression during gastrulation marks the developing

mesoderm (Scialdone et al., 2016). Therefore, several studies have used KDR as a marker to isolate mesoderm cells from hESCs (Table 3). Mesoderm populations that express KDR represent a valuable source for generating hematopoietic and endothelial lineages (Sriram et al., 2015). In adults, KDR might be involved in mitosis, vascular permeability and angiogenesis (Chen et al., 2019). Overexpression of KDR is associated with endothelial cell malignancies, for which a wide range of potential KDR inhibitors are reported for the management of cancer (Modi and Kulkarni, 2019).

Mix Paired-like Homeobox (MIXL1)

MIXL1 is another marker used for identifying the differentiation of pluripotent cells into mesoderm cells (Table 3). MIXL1 is markedly needed in mesoderm formation in early development (Wolfe and Downs, 2014). However, MIXL1 is required for the development of endoderm, for which studies have used it as an endoderm marker (Table 2). MIXL1 was also shown to be expressed in mesendodermal precursor cells (Alexeeva et al., 2016). Therefore, MIXL1 might not be a useful candidate to be used as a mesoderm marker.

Conclusion

A wide range of markers have been reported in the literature as indicative of the three germ layers. However, there are inconsistencies between these studies due to the lack of validation protocols and a standard set of markers for the early trilineage specification. Based on the recent literature, the most commonly used ectoderm markers NES and TUBB3, endoderm markers FOXA2, CXCR4 and GATA4, and mesoderm marker MIXL1 are also found to be expressed in different germ layer cells. This may render them unreliable for the use as early differentiation markers. The current review highlights the need for further development of validation protocols to organize the use of early differentiation markers, which may help generate efficient cells to be used for treating degenerative diseases and in drug screening. Investigators should be cautious in selecting the early differentiation markers until definitive standard assays have been established.

Author Disclosure Statement

The author declares no competing financial interests exist.

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