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Disease modeling using human induced pluripotent stem cells: Lessons from the liver☆

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Abstract

Human pluripotent stem cells (hPSCs) have the capacity to differentiate into any of the hundreds of distinct cell types that comprise the human body. This unique characteristic has resulted in considerable interest in the field of regenerative medicine, given the potential for these cells to be used to protect, repair, or replace diseased, injured, and aged cells within the human body. In addition to their potential in therapeutics, hPSCs can be used to study the earliest stages of human development and to provide a platform for both drug screening and disease modeling using human cells. Recently, the description of human induced pluripotent stem cells (hIPSCs) has allowed the field of disease modeling to become far more accessible and physiologically relevant, as pluripotent cells can be generated from patients of any genetic background. Disease models derived from hIPSCs that manifest cellular disease phenotypes have been established to study several monogenic diseases; furthermore, hIPSCs can be used for phenotype-based drug screens to investigate complex diseases for which the underlying genetic mechanism is unknown. As a result, the use of stem cells as research tools has seen an unprecedented growth within the last decade as researchers look for *in vitro* disease models which closely mimic *in vivo* responses in humans. Here, we discuss the beginnings of hPSCs, starting with isolation of human embryonic stem cells, moving into the development and optimization of hIPSC technology, and ending with the application of hIPSCs towards disease modeling and drug screening applications, with specific examples highlighting the modeling of inherited metabolic disorders of the liver. This article is part of a Special Issue entitled Linking transcription to physiology in lipodomics.

Keywords

hIPSC; Liver; Stem cells; Lipid disorders; Human development; Disease modeling

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1. Introduction

1.1. Stem cells and the era of regenerative medicine Introduction

1.1.1. Degenerative disease—Western society has seen a dramatic increase in life expectancy over the last century due to the development of vaccines, antibiotics, improved sanitation, and increased general public health awareness. This increase in life expectancy due to decreased occurrence and severity of infectious disease has now unmasked an increase in debilitating illness and morbidity due to degenerative diseases. Degenerative disease is characterized as a progressive deterioration of the structure and/or function of an organ or tissue over time. These diseases are recognized as major health problems that are either currently affecting or will affect the general population in the near future. Additionally, increased incidence of degenerative disease is also of major economic concern as patient management and care, as well as the associated costs of infrastructure, can deliver a high financial burden. Diseases such as Alzheimer's and Parkinson's disease, motor neuron disease, multiple sclerosis, diabetes, kidney, liver, and heart disease, as well as blindness and cancers of many types, are all, at their most basic level, due to loss of environmental and cellular homeostasis of the resident stem cell pools in these tissues. This loss of stem cells ultimately leads to hypoplasia, a gradual loss of a population or populations of cells that is unable to be replaced, leaving the organ or tissue structurally and functionally deficient. Unfortunately, current treatments for many degenerative diseases simply address symptoms, reducing their severity or the speed of their onset. Presently, there are no reliable preventative or curative treatments for degenerative diseases other than whole organ transplantation, which often has limited success and use due to immune rejection and insufficient supply of suitable donor organs compared to demand.

1.1.2. Self-renewal and pluripotency—Human pluripotent stem cells (hPSCs) possess two remarkable cellular characteristics that set them apart from all other stem cells and make them an ideal candidate for regenerative medicine applications: the properties of “self-renewal” and “pluripotency” (Fig. 1). Self-renewal refers to the ability of these cells to make identical copies of themselves indefinitely, without developing chromosomal abnormalities or undergoing growth arrest. Pluripotency refers to the ability of these cells to differentiate into any cell of the human body, following the natural path of human embryonic development, when given the appropriate signals to do so. Since hPSCs were first isolated in 1998, numerous studies have shown that these cells are indeed able to form extraembryonic tissues such as trophectoderm [1,2], the three primary germ layers; ectoderm, mesoderm and endoderm [3], and a myriad of cell types derived from these primary germ layers, including from ectoderm neurons [4], keratinocytes [5], retinal epithelium [6], from the mesoderm skeletal muscle [7], cardiomyocytes [8], hematopoietic cells [9], vascular smooth muscle [10,11], osteoblasts [12], chondrocytes [13], and cells of the kidney [14], and from the endoderm liver [15], lung [16], and pancreas [17]. Therefore, it is self-evident that hPSCs represent a viable source of cells to combat degenerative disease in society.

2. A brief history of pluripotent stem cells

The concept of a “stem cell” is not a new one. Work in the hematopoietic system identified populations of stem cells that were capable of producing all of the cells that comprise the

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human blood system [18]. Following this discovery, many other stem cell populations have been identified and are implicated in replenishing various tissues over the lifetime of an organism [19]. However, it was observations within the tissue mass of a certain type of rare tumor known as a teratoma that gave researchers the first hint that a stem cell capable of producing *all* of the tissues found in an adult organism might exist.

2.1. Teratomas and teratocarcinomas

A teratoma is a rare tumor that forms in the gonads of a wide number of vertebrates including mice and humans [20]. Teratomas contain cells representative of all three germ layers that are arranged within the tumor mass in a random and disorganized manner. These tumors can be either benign or malignant, the latter known as a teratocarcinoma [21]. The cells that comprise the differentiated population of the teratocarcinoma are generally not malignant; however, certain cells responsible for the malignant properties of the tumor, *i.e*. the ability to initiate new tumor formation and repopulate the entire mass of the tumor, are called embryonic carcinoma (EC) cells and represent a dysregulated form of cancerous stem cells [22].

Both human and mouse EC cells have subsequently been isolated and cultured *in vitro*, each with their own unique and shared growth and differentiation characteristics [20]. It has been demonstrated in the mouse that EC cells can be isolated from teratocarcinomas, cultured *in vitro*, and transplanted back into mice to form new teratomas [23]. These cells are able to be cultured *in vitro* using methodologies reserved for other malignant cell lines, demonstrate a loss of contact growth inhibition, can be established as permanent cultures, and most importantly demonstrate the ability to self-renew and differentiate into a wide variety of cell types [24,25] (Table 1). Extensive studies of EC cells led to the development of culture methodologies that would eventually result in the isolation and culture of mouse embryonic stem cells (mESCs), which in turn would lead to the isolation of the first human embryonic stem cells (hESCs).

3. Towards the derivation of human embryonic stem cells

3.1. Mammalian embryonic development

To appreciate where the regenerative power within each hPSC originates, it is important to understand the origin and purpose of these cells during human development. Development from a single cell to a fully formed neonate proceeds through many complex developmental stages that are completed in as little as 19 days in the mouse [26], 280 days in humans [27], or up to 640 days in the African elephant [28]. Despite the differences in developmental and gestational periods, all placental mammals share a very similar developmental program prior to uterine implantation.

Following fertilization, the resulting single cell, the zygote, undergoes a series of cell divisions that divide the large cell into a cluster of smaller cells called the morula [29]. The morula consists of approximately 4–16 cells surrounded by a glycoprotein membrane called the zona pellucida [30]. As development continues, the cells of the morula, called blastomeres, continue to divide and form a central fluid filled cavity called the blastocoel [31]. At this stage, the cell mass is called the blastocyst and consists of approximately 40–

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150 cells [32]. The blastocyst then undergoes a process called hatching. During this stage, the blastocyst moves or hatches through an eroded hole in the zona pellucida, exposing the blastocyst to the uterine environment [33,34] (Fig. 2).

The blastocyst now consists of two distinguishable cell types, an outer cell layer called the trophoblast and a small group of cells within the blastocyst called the inner-cell mass (ICM) [35]. It is from the ICM at this stage in mammalian development that embryonic stem cells can be isolated [3,36,37]. This entire process occurs during migration from the distal fallopian tube into the uterus and is referred to as the “pre-implantation” stage. An analogous process can also be carried out *in vitro* using techniques pioneered by *in-vitro* fertilization (IVF) to obtain viable blastocysts. Under normal developmental conditions, it is the cells of the ICM that eventually give rise to all the cells in the adult organism. The ability to allow human development to proceed to the blastocyst stage *in vitro* has allowed researchers access to the ICM and paved the way for the first isolation of a human ICM (Fig. 2).

3.2. First isolation of hESC

The isolation and establishment of hESC lines from the ICM are challenging procedures that were first accomplished by James Thomson in 1998 [3]. The methodology used then and still used today is very similar to the methodologies used to isolate the very first mESC lines in 1981. The procedure involves removing the trophectoderm layer of the blastocyst, usually via mechanical dissection or complement-mediated immunosurgery, to reveal the inner cell mass (ICM), which is then plated onto a layer of mitotically inactivated feeder cells, most commonly mouse embryonic fibroblasts (MEFs) [38]. The isolated ICM is allowed to expand for several days before mechanical dissociation and transfer onto a fresh layer of feeder cells. This process can be repeated indefinitely and it is at this point that the isolated ICM is regarded as a stable hESC line.

3.3. Basic hESC characterization

To be considered a pluripotent hESC, a cell must have originated from a pluripotent cell population such as the ICM of a preimplantation embryo, must have and maintain a normal karyotype, must be immortal and capable of indefinite passaging, and must demonstrate the ability to differentiate into cells representative of all three germ layers, either via *in vivo* teratoma formation or *in vitro* differentiation [39–41]. Within the mESC system, the ultimate test of pluripotency is the generation of chimeric mice. This is achieved by injecting cultured mESCs into preimplantation mouse blastocysts. The contribution of the mESC line to adult tissues representative of all three germ layers, including germline transmission, is considered unequivocal evidence for the pluripotent nature of these cells. Due to obvious ethical considerations, this experiment cannot be performed using hESCs; therefore, the most reliable test of hESC pluripotency remains the ability to form teratomas exhibiting cells and tissues representative of all three germ layers.

Once isolated and plated onto a feeder layer, hESCs develop very distinct morphological characteristics such as a high nucleus-tocytoplasmic ratio, with each cell having one or more prominent nucleoli [42]. Undifferentiated hESCs grow in very compact colonies that are

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typically multi-layered, have well defined colony borders, and can be easily distinguished from the feeder layer (Fig. 3A, B). More importantly, hESCs display a unique expression profile of intracellular and surface markers of pluripotency such as strong expression of the transcription factors POU5F1, SOX2, and NANOG, in addition to the cell surface markers TRA-1–60, stage specific embryonic antigen (SSEA) 3, and SSEA-4 [43,44] (Fig. 3C). Interestingly, this pattern of expression has also been observed in human blastocysts, suggesting that hESCs derived from the pre-implantation ICM retain some characteristics of the *in vivo* ICM [45].

4. A brief history of induced pluripotent stem cells

The first human induced pluripotent stem cells (hIPSCs) were described by Shinya Yamanaka and James Thomson in 2007, less than ten years after the isolation of the first hESCs. However, the understanding required to develop this revolutionary cell type began more 50 years earlier with the work of Thomas King and Joseph Briggs, in Philadelphia USA, as well as John Gurdon, in Cambridge UK, who independently were able to show that the oocyte contains factors that can restore or reprogram the developmental potential of an adult somatic cell within tadpoles [46]. This work eventually gave rise to somatic cell nuclear transfer (SCNT), also known as “cloning”, the most well known example being “Dolly” the sheep. Dolly was born in 1996 and was the first mammal to be successfully cloned, demonstrating that oocyte derived reprogramming factors are capable of restoring the developmental potential of mammalian cells as well [47]. More recently, human skin fibroblasts have been reprogrammed using SCNT and viable stem cell lines were generated from the resulting blastocysts [48]. The principle of SCNT formed the basis for the study of Shinya Yamanka, who was able to successfully reprogram the first mouse somatic cells into PSCs in 2006 and subsequently, along with James Thomson, apply those findings to the human system a year later in 2007 [49–51].

4.1. The Yamanaka factors & IPSCs

Beginning with a small library of 24 candidate genes shown to be intimately linked with the mouse pluripotent state, Yamanaka and colleagues began expressing these 24 genes in mouse fibroblasts. The fibroblasts used for this work carried the β-galactosidase gene as well as neomycin resistance expressed in the Fbx15 locus. Fbx15 was used since its expression is correlated with pluripotency in the mouse system; although, it is not explicitly necessary for the pluripotent state. When fibroblasts transfected with the 24 factors were grown in tissue culture medium specific for pluripotency and were supplemented with the antibiotic G418, several drug-resistant colonies arose that displayed many characteristics of pluripotency including pluripotent morphology, formation of tightly packed colonies, high nuclear to cytoplasm ratio, high proliferation rate, and expression of pluripotent specific gene signatures. Through a process of elimination, the 24 factors were gradually reduced to four genes: POU5F1 (Oct3/4), SOX2, KLF4, and c-Myc, together known as the Yamanaka factors. POU5F1 and KLF4 proved to be essential for reprogramming, while SOX2 and c-Myc affected the efficiency of the reprogramming process. While these cells were able to form embryoid bodies as well as teratomas containing all three germ layers, these cells could not form chimeric mice when injected into a donor mouse blastocyst. However, this problem

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was overcome less than twelve months later by Yamanaka and two other independent research groups when they used NANOG to select reprogrammed cells rather than Fbx15. They subsequently demonstrated that these induced pluripotent cells could form chimeric mice and were germ line competent, the most thorough tests of pluripotent potential [49].

4.2. Human IPSCs

IPSCs from the mouse were an instant sensation, and the feat was quickly reproduced using human fibroblasts twelve months later, again by Shinya Yamanaka as well as James Thomson. Interestingly, the Yamanaka group demonstrated that the same four factors used to reprogram mouse fibroblast were sufficient to reprogram human fibroblast. The Thomson lab demonstrated that some of the Yamanaka factors could be substituted with other factors with similar functions. Thomson achieved successful reprogramming of human fibroblasts using NANOG and LIN28 instead of c-Myc and KLF4. The obvious ethical and moral considerations of human embryo work preclude testing for chimera formation and germ line transmission of hIPSCs; however, the reprogrammed HIPSCs share all of the same features of the embryonic equivalents [50–52]. Human ESCs and hIPSCs have distinctly different origins yet appear almost identical in their capacity to differentiate into all cell types of the human body. Additionally, hIPSCs avoid the ethical considerations of utilizing hESCs, and thus, have revolutionized regenerative medicine and current approaches to treating and curing diseases (Fig. 4). This discovery was recognized by the award of the Nobel Prize to Sir John Gurdon and Shinya Yamanaka in 2012 for their groundbreaking work on reprogramming leading to IPSC technology.

5. Embryonic development, HIPSCs and disease modeling

One of the most exciting applications of hIPSC technology is the modeling of diseases *in vitro*. Since the starting material for reprogramming is generally skin fibroblasts taken from a simple punch biopsy, the generation of hIPSCs from any desired genetic background has become easily accessible and almost routine (Fig. 4).

Using HIPSCs for disease modeling and drug screening relies on three important conditions: (1) the ability to differentiate HIPSCs into cells representative of the disease organ or tissue, (2) being able to faithfully reproduce the key aspects of the disease and (3) to be able to qualitatively or quantitatively measure disease progression and/or regression. Each of these three aspects is difficult to achieve; however, the field of disease modeling has grown significantly over the last five years, with many disorders having been successfully recapitulated *in vitro* [53–59]. Most of these studies have focused on monogenetic disorders that display strong phenotypes *in vitro;* however, the aim of the field is to develop this technology to model more complex disorders.

5.1. Differentiation of hIPSCs into complex tissues and cells types

The most challenging aspect of disease modeling is often the development of robust differentiation protocols that produce pure populations of functional cells that accurately recapitulate fundamental characteristics of the diseased tissue. Our group has spent much time modeling inherited metabolic disorders of the liver using hPSCs, and a very large

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proportion of that time was initially invested in developing protocols which reliably produce functional adult hepatocytes which mirror their *in vivo* counterparts [15,54,60,61]. The main approach to this task, in the absence of human developmental data, is to model the differentiation protocol based on other developmental systems, primarily from knowledge obtained from the mouse. Understanding the major landmarks of mouse development, which in many ways parallels human development, allows one to intelligently approximate the signaling pathways and gene expression profiles that should exist at different stages of liver specification and maturation.

5.2. Embryonic development from the blastocyst to specification of the liver

To understand how different tissue types might be obtained using hIPSCs, understanding the post-blastocyst stages of development is essential. Prior to implantation, the ICM has no apparent organizational structure (Fig. 5A), yet by the time of implantation, the ICM separates from the trophoblast, forming a cavity called the amniotic cavity (Fig. 5B). The ICM subsequently flattens into an oval shaped disk that is two cellular layers thick, known as the embryonic disk. The upper layer is called the epiblast and the lower layer, the hypoblast (Fig. 5C). The embryo develops entirely from the epiblast while the hypoblast cells go on to form the yolk sac [62].

The next stage of development, called gastrulation, involves cells of the epiblast forming a thickened ridge of cells on the embryonic disk, called the primitive streak [63] (Fig. 5C). As cells begin to move toward the primitive streak, some begin to move inward, resulting in three distinct layers of cells. These three layers of cells, ectoderm, mesoderm, and endoderm, are called the germ layers and will form all of the cell types found in the adult animal [63,64] (Fig. 5D, E). The formation of the primitive streak and the patterning of the three embryonic germ layers involve the exposure of cells to signaling pathways such as Activin/ Nodal, BMP, FGF, and Wnt, during which time the combination of each of these factors, as well as the duration of signaling, is important. Translating this process to an *in vitro* setting can be quite difficult; however, there are now many established protocols that are routinely used to produce each of the three embryonic germ layers from HIPSCs [65–68].

As the newly specified ‘naive’ endoderm migrates through the primitive streak, it displaces underlying cells comprised of visceral endoderm and migrates along the overlying mesoderm layer. At this stage, the endoderm is a two-dimensional sheet of cells that can be divided into anterior and posterior domains [69]. In the mouse, initially the endoderm is located on the outer most layer of the embryo; however, as development continues, the embryo undergoes a complex series of movements and rotations that result in the inversion of the germ layers, causing the endoderm to become the innermost layer of cells [69,70]. Throughout this process, the flattened sheet of endodermal cells continues to expand, migrate, and eventually form the anterior, posterior, and lateral domains of this expanding epithelial sheet, finally converging on each other to form a closed primitive gut tube. The primitive gut tube is broadly defined as having three domains: the foregut, marked by SOX2 expression, the midgut, marked by PDX1 expression, and the hindgut, marked by CDX expression [71,72]. Initially, the anterior and posterior regions of the primitive gut are

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'plastic' and when given the appropriate signal the hindgut domain is capable of initiating the foregut differentiation program [73]. As development continues, the hindgut domain develops into the large intestine, the midgut into the small intestine, while the foregut produces the thyroid, oesophagus, lungs, liver, pancreas, the biliary tree, and stomach. The patterning of the foregut into such a diverse array of cells is controlled by a complex network of many soluble factors secreted in part by the overlying mesoderm layer (Fig. 5F, G).

The cardiac mesoderm (CM) and the septum transversum mesenchyme (STM) play a critical inductive role in liver development [74]. The CM is responsible for creating an FGF gradient along the foregut, while the STM secretes BMPs, which together with FGFs, induce liver bud formation [75] (Fig. 6A). Varying concentrations and ratios of FGFs and BMPS along with other soluble factors, such as Wnt and retinoic acid (RA), influence the patterning of the entire length of the foregut [76] (Fig. 6B). The first inductive signals that the foregut endoderm receives occur while the foregut is still a flattened sheet of cells [77]. At this point, several domains of the future foregut have been specified as liver, and as the primitive gut closes to form the gut tube, it allows the cells committed to the hepatic lineage to form a liver bud.

Once the specified hepatic domains come together in the newly formed gut tube, they begin to delaminate and invade the surrounding mesenchymal tissue. This process is marked by the expression of transcription factors such as HHEX, GATA4, GATA6, HNF6, and PROX1 [78]. Similarly, the liver bud stage is defined by expression of AFP, TTR, and ALB within the hepatic bud [75]. At this developmental time point, the cells within the hepatic bud are referred to as hepatoblasts, owing to their ability to produce both hepatocytes as well as cholagiocytes. Several genes have been shown to be important for formation and progression of liver bud development such as HHEX. HHEX null mouse hepatoblasts fail to migrate into the septum transversum mesenchyme, while PROX1 and TBX3 knockouts result in various developmental defects including an inability to delaminate and hindered cell proliferation.

As invasion into the surrounding mesenchyme continues, the surrounding basement membrane is degraded and invaded by chords of hepatoblasts marked by GATA4 and HNF4A expression. At the same time, angiogenesis and vasculogenesis proceed as angioblasts and endothelial cells permeate the liver bud. The hepatoblasts differentiate into either into hepatocytes or billiary epithelial cells in a process that is not well understood, but is known to be mediated in some way by the notch signaling pathway. How the cells of the embryonic liver organize themselves into the complex hepatic architecture that is crucial for normal liver functionality is only now beginning to be understood.

5.3. Endoderm and hepatic differentiation in vitro

Translating mammalian development from its *in vivo* context to a dish in the laboratory is challenging to say the least. Gastrulation is driven by a diverse number of signaling pathways acting in both spatial and temporal gradients. We have been able to recapitulate this developmental system using combinations of Activin-A, BMP4, bFGF, Wnt, and PI3K inhibition using the small molecule LY294002 [79,80] (Fig. 7A). Treating undifferentiated

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pluripotent cells for three days with these factors induces a population of cells that transiently upregulate the mesendoderm markers T and MIXL1, downregulate the pluripotency markers POU5F1, NANOG, and SOX2, and after three days, upregulate endoderm markers such as SOX17, GATA4, and CXCR4. This population of cells shares an expression profile very similar to definitive endoderm (DE) isolated *in vivo*. We have also demonstrated the developmental competency of this population by showing that treating our DE population with Activin-A produces a SOX2 expressing population that is more similar to the *in vivo* foregut, while treatment with Wnt produces a CDX2 positive population that is able to produce intestinal epithelium of the hindgut [67,81].

After treating the DE with Activin-A we induce a gene expression profile that more closely resembles the primitive foregut, marked by expression of SOX17, CXCR4, GATA4, HNF4A, and anterior HOX genes. This population of foregut endoderm is now primed to respond to multiple signals to produce all cell types of the foregut. Treatment of these foregut cells with a combination of FGF10 and BMP4 causes the cells to upregulate hepatic genes such as AFP, ALB, TBX3, PROX1, HHEX and HNF6. This population of cells, patterned to hepatic endoderm, can be matured into more adult like hepatocytes using a combination of OSM and HGF. The resulting population expresses high levels of ALB, AAT, APOF, LDLR, and HNF4A (Fig. 7B, C, D), as well as moderate levels of cytochrome P450 enzymes such as CYP3A4, CYP1A1, and CYP1A2 [15,17,54,67,82].

In addition to gene expression profiles generated by quantitative PCR, the validity of the cells produced can be demonstrated through immunocytochemistry, western blotting, flow cytometric analysis (FCA), functional assays, and *in vivo* injection to demonstrate compatibility with similar *in vivo* biological environments. With regard to FCA, we aim to have at least 80% of the cells positive for markers of DE, hepatic endoderm, and mature hepatocytes at each of the corresponding stages to ensure that quantitative functional analysis of the endpoint population is not hindered by a significant proportion of contaminating cells that could possibly introduce false positive or negative results to drug screening outcomes.

Once a protocol is established that reliably produces a homogeneous population of target cells, hIPSCs from patients carrying metabolic disorders that should present a phenotype within the differentiated cell type can be differentiated using the optimized protocol. The next important and critical step after these patient derived hIPSCs are differentiated is to validate the disease phenotype and compare to known *in vivo* data.

5.4. Modeling familial hypercholesterolemia (FH)

Familial hypercholesterolemia is a genetic disorder characterized by high levels of low density lipoprotein (LDL) in the blood, affecting as many as 1 in 500 births. There are many mutations causing FH; however, the most severe are caused by mutations in the LDL receptor (LDLR), normally expressed by hepatocytes in the liver that are responsible for uptake and metabolism of LDL from the blood. As a result of insufficient clearance of LDL from the blood, heterozygous patients often develop premature cardiovascular disease in their 30s–40s, while homozygous patients will sometimes develop cardiovascular disease before their teenage years. Heterozygous patients are less complicated to treat and can

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respond well to statin therapies; however, homozygous patients do not respond well to any known treatment and require a liver transplant to correct the disease.

We have successfully modelled FH *in vitro*, reproducing the phenotype of deficient receptor activity and LDL metabolism [54]. To do so, skin biopsies were taken from patients who were homozygous for the LDLR mutation, which results in receptor activity as low as 2% when compared to WT cells. The skin fibroblasts were then reprogrammed using the Yamanaka factors and established as induced pluripotent stem cell lines (LDLR-hIPSCs) (Fig. 4). The stable LDLR-hIPSC lines were then differentiated into adult hepatocytes using our hepatic differentiation protocol along with WT hIPSCs. The LDLR-hIPSCs were able to produce endoderm, foregut, hepatic endoderm, and finally adult hepatic cells. In the case of FH, the phenotype arises in mature hepatocytes, and all other functions of the liver should appear normal. We were able to demonstrate this in our system, showing that all gene expression profiles, including expression of LDLR, were similar to wild type cells, and that other functional outputs such as albumin secretion, α-1-antitrypsin secretion, and cytochrome p450 metabolism were within the normal range of wild type cells. Genome sequencing confirmed the presence of the mutation within LDLR gene and QPCR analysis along with western blotting confirmed that the gene was indeed expressed within the hepatic cells both at the mRNA and protein levels. However, by determining the rate of LDL uptake from the culture medium, we were clearly able to see the FH phenotype. To quantify LDL uptake and demonstrate the FH disease phenotype, we used a simple LDL uptake assay. These types of assays employ LDL conjugated to a fluorescent dye so that cells that express LDLR and are able to bind LDL can be visualized using fluorescent microscopy techniques and quantified using FCA. Using FCA and comparing binding and uptake of LDL to WT cells, we could see a quantitative difference in the amount of bound LDL between the two cell types (Fig. 8A, B). Having validated the cellular phenotype within this system and having developed a reliable functional readout, it is possible to utilize high-throughput screening of novel therapies and small molecules that may be able to reverse the phenotype and provide relief for sufferers of FH.

5.5. Validating targeted mutation correction using alpha-1-antitrypsin deficient IPSCs

Alpha 1 -antitrypsin deficiency (A1AD) is a genetic disorder affecting nearly 1:3000 individuals in which a point mutation in the alpha 1-antitrypsin (AAT) gene causes a non-functional polymeric version of the normally monomeric AAT protein. AAT is mainly produced by hepatocytes and released into the blood, but also is expressed to a lesser degree in the lungs and pancreas, and functions as a systemic protease inhibitor. Symptoms arise due to local protease overactivity and vary in severity depending on whether the patient has one or two copies of the mutated allele. A1AD symptoms occur in a wide range of organs and manifest as a variety of conditions including psoriasis of the skin, cirrhosis of the liver, inflammatory bowel disease (IBD), and chronic obstructive pulmonary disease (COPD), among others. A1AD is of particular interest for disease modeling because (1) the disease phenotype has been causally associated with both a cell type and specific mutation, and (2) reliable differentiation protocols exist for producing mature hepatocytes from patient derived HIPSCs.

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In recent years, hIPSC lines have been derived from patients who possess the mutant AAT protein (A1ATD-hIPSCs) and subsequently have been terminally differentiated into hepatocytes [54]. These cells have been thoroughly characterized and compared to primary controls and, as expected, exhibit the polymeric AAT phenotype associated with the disease (Fig. 9). Having validated the ability of these cells to recapitulate the *in vivo* disease phenotype in an *in vitro* setting, the next step was to evaluate potential ameliorative or curative therapies for the disorder using these cells as a metric for efficacy. Our group decided use a targeted gene therapy approach to fix the point mutation in order to eliminate the non-functional polymeric protein. To do so, zinc finger nucleases (ZFNs) and piggyback transposons were used to excise the mutant allele and replace it with a normal variant [61]. The resultant corrected A1ATD-hIPSCs were differentiated into mature hepatocytes, and much to our delight, expressed normal levels of functional monomeric AAT and undetectable levels of the mutant form (Fig. 9). Eventually, such genetically corrected cells could be reintroduced to the patient in order to alleviate symptoms caused by lack of functional AAT in the bloodstream.

This example highlights the power of disease specific hIPSCs and shows how researchers can go full circle from investigating a disease in an *in vitro* setting, to testing and validating novel therapeutics on human cells that are actively manifesting a disease phenotype, to utilizing corrected autologous cells as a curative therapy, all by using hIPSCs.

6. Towards the clinic: current limitations and future directions

Generation of patient specific stem cells that can be used to deliver personalized therapies back to the patient remains the ultimate goal for regenerative medicine. Initial barriers that may hinder or delay the application of these cells include the development of robust reprogramming and differentiation protocols using clinically approved factors, as well as developing accurate, standardized assays that can be used to assess the quality of the desired outputs. Even so, these problems may be far less complicated than those that present in delivering these cells back to the patient in a safe and efficacious manner.

6.1. Reprogramming

Until recently, HIPSCs were derived using dermal skin fibroblasts and retroviral or lentiviral vectors encoding the four Yamanaka factors. Lentiviral mediated reprogramming works via the random incorporation of the viral genetic element into the genome. Recently non-integrative methods have been demonstrated, such as Sendai virus mediated reprogramming; however, retroviral reprogramming still remains the most common approach. Random integration renders the genome of each cell unique, and although these viral elements should be silenced once reprogramming has occurred, even small levels of reactivation during differentiation have the potential to dramatically affect the behaviour of the cell. Random integration of viral elements into genome can disrupt the correct regulation of genes important for cellular phenotype and result in misleading outcomes and observations. Non integrating vectors, excisable vectors, Sendai viruses, mRNA, microRNA, and small molecule mediated reprogramming have all been reported and are aimed at reducing the effects integrating viruses can have on the genome; however, these techniques are often very

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expensive or exhibit far lower reprogramming efficiency compared to the retroviral standard.

6.2. Epigenetic memory

Selecting a fully reprogrammed cell in a culture dish full of fibroblasts, partially reprogrammed cells, and colonies of cells displaying several different growth and morphological characteristics can be challenging. The current ‘state of the art’ is an experienced practitioner identifying cells growing that have the correct cellular morphology and growth characteristics that is forming larger colonies of cells with those same morphological and growth characteristics. Validation that these cells indeed express endogenous POU5F1, NANOG, and SOX2, and have subsequently silenced the reprogramming vectors, is typically enough to qualify cells as successfully reprogrammed. With this relatively low level of characterization, differences in growth characteristics and differentiation capacities is routinely observed, suggesting there are other important considerations when identifying the most suitable cell lines to proceed with disease modeling. The epigenetic state of hIPSCs is beginning to be more precisely understood, and the critical influence it has on reprogrammed cells is being more fully appreciated. It is now recognized that many HIPSCs retain an epigenetic memory of their donor cells. The epigenetic profile of HIPSCs is very similar to that of hESC; however, there are some specific epigenetic characteristics that are only seen in HIPSCs and may account for variability seen between hIPSC and hESC lines. The inefficient clearing of epigenetic memory in HIPSCs affects both cell fate during differentiation and the ability of the cell to perform functional tasks when fully differentiated into an adult cell. The role of epigenetic status is not fully understood but most certainly influences HIPSCs.

6.3. Disease selection

Not all diseases are amenable to disease modeling using HIPSCs. Ideally the disease to be studied should manifest at the cellular level and have a phenotype that can be clearly demonstrated using tissue culture techniques. As many differentiation protocols are yet unable to produce the full spectrum of adult functionalities, diseases that manifest early during development and require less complex and lengthy differentiations are generally much easier to study. Similarly, disease resulting in loss of expression or misexpression of genes that can be quantified easily at the protein level using western blotting and or immunocytochemistry and FCA also present a system far easier to interrogate. Diseases such as Alzheimer's and Parkinson's, while very appealing due to their increasing incidence in the population, provide a seemingly overwhelming challenge as links between cellular disease phenotype and clinical presentation and pathology are poorly described.

6.4. Appropriate positive controls

Assessing the successful conversion of undifferentiated HIPSCs from their pluripotent state into that of a functional adult cell is impossible to fully evaluate without the use of an appropriately matched primary adult cell from both WT and diseased donors. Additionally, an appropriate wild-type hIPSC differentiated into the cell type of interest should also be considered as another benchmark when evaluating differentiation success, attainment of adult functionalities, and reproduction of a disease phenotype in disease model HIPSCs.

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Finding appropriate controls is often a difficult challenge as some cell types are rare, difficult to isolate, and/or expensive to purchase from commercial sources. Furthermore, functionality of some primary cells can quickly and severely diminish once the cell is removed from its *in vivo* environment, leaving researchers without a meaningful positive control.

7. Conclusion

Induced pluripotent stem cells have the potential to make a significant impact on both the way we study disease and how we will treat diseases in the near future. As we have discussed, there are several obstacles that need to be overcome in order to produce a disease model that can accurately and consistently reproduce a particular disease phenotype and allow for accurate prediction of the efficacy and toxicology of novel therapeutics. Ultimately, our ability to consistently and faithfully differentiate hIPSC lines into desired cell types is likely to have the most dramatic effect on the reliability of the system. As our understanding of human development and the intrinsic and extrinsic factors that drive tissue and organ development evolves, we will produce ever more reliable cells for disease modeling and transplantation purposes.

Reprogramming itself is largely an unknown mechanism that we are only beginning to understand. Reprogramming clearly introduces variability into the equation, which currently can only be minimized through the thorough use of multiple cell lines harboring the same genetic mutation coupled with use of matched primary controls. Variability should, however, continue to become less of an obstacle as we continue to explore new reprogramming technologies that involve both non-integrative and non-viral methodologies.

Researchers, clinicians, and the pharmaceutical industry of the past could not have predicted that libraries of HIPSCs comprising the entire spectrum of human disease could be used to differentiate into any cell of the human body so that almost any disease of any tissue could be studied. As we continue to develop this system, it seems hard to envisage a way we could move forward without them.

Acknowledgments

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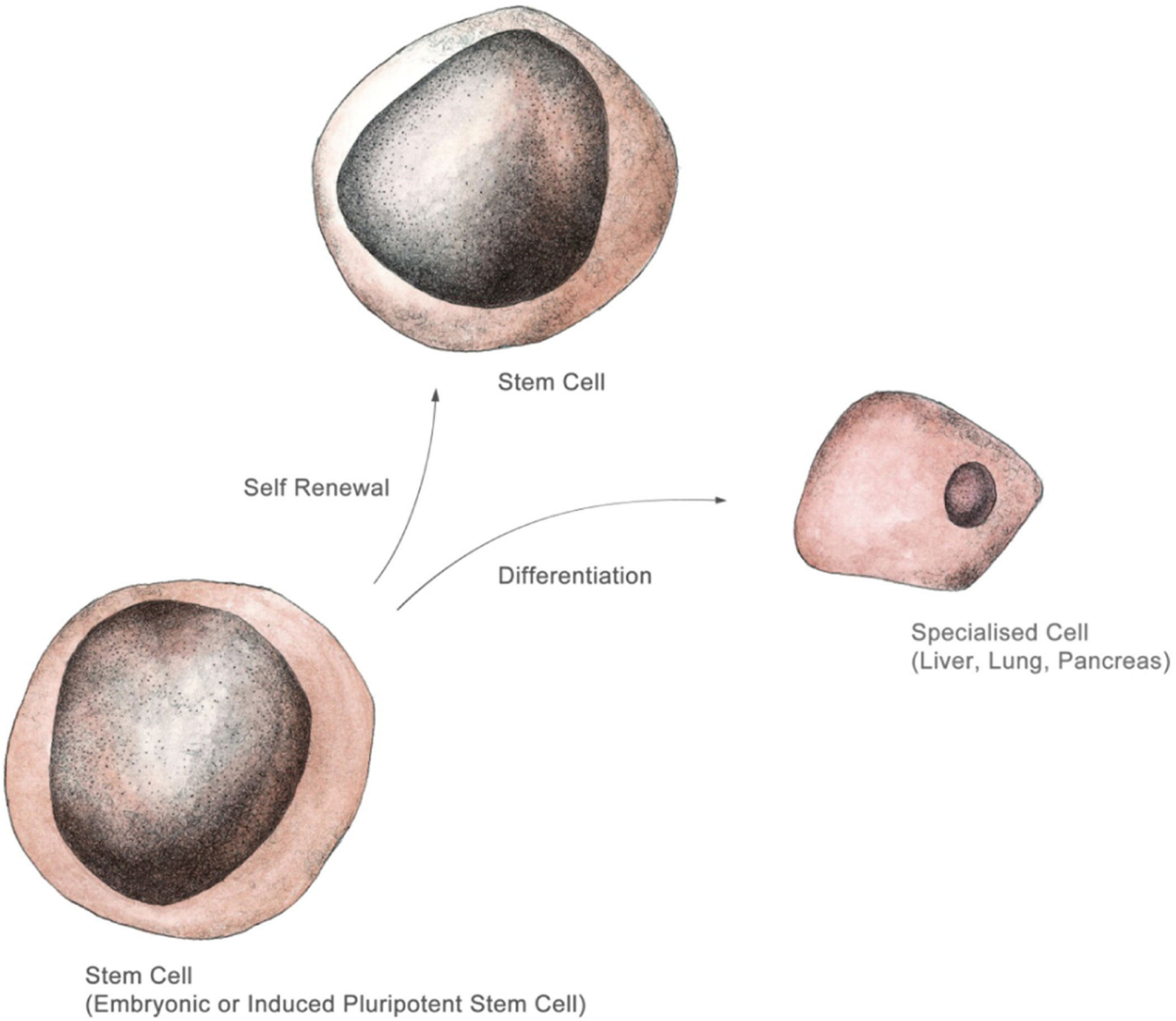

Fig. 1.

Self-renewal and pluripotency. Human pluripotent cells are characterized by their unique ability to self-renew, making unlimited identical copies of themselves while retaining their pluripotency, the capacity to differentiate into more specialized cells.

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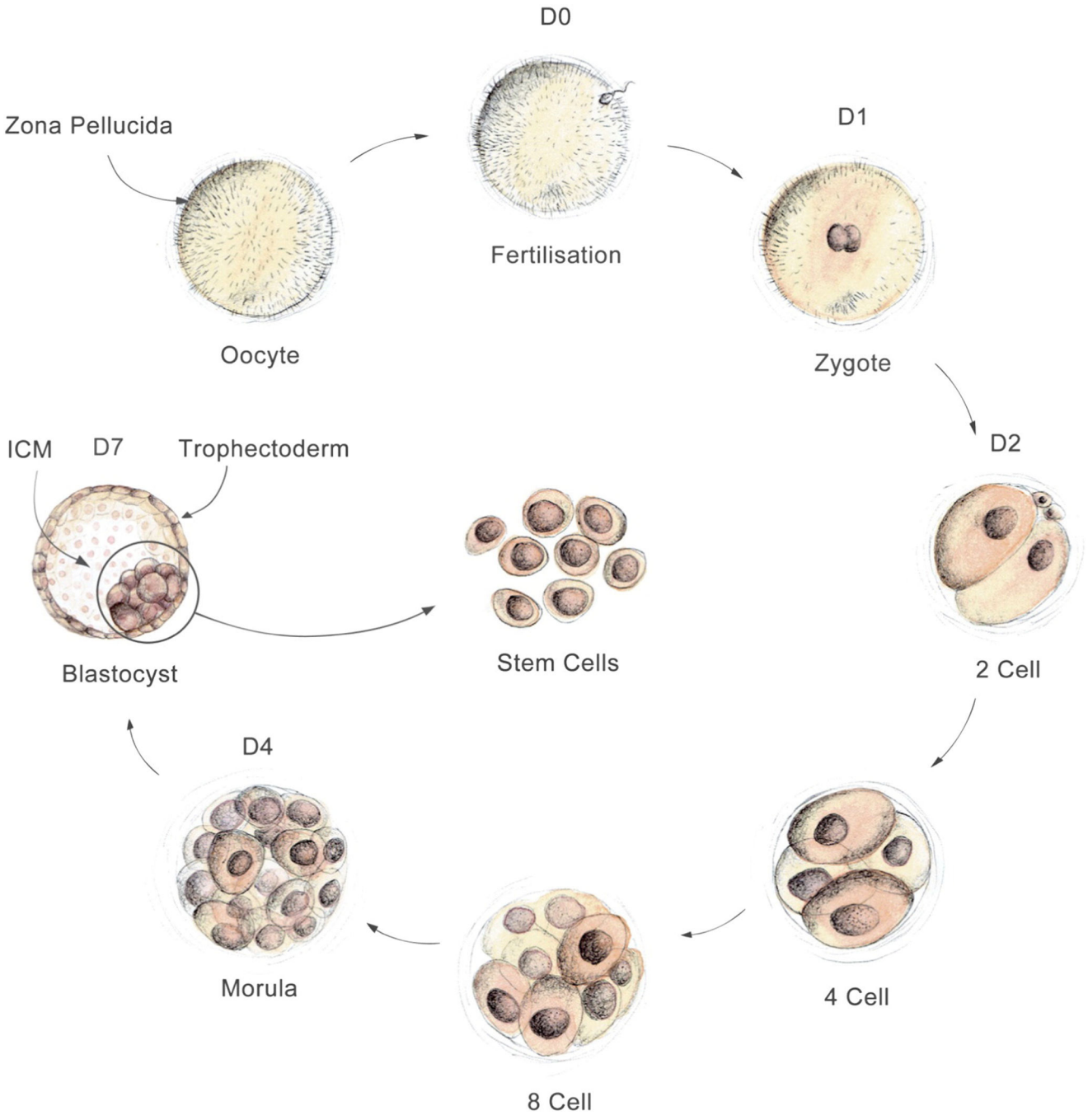

Fig. 2.

Mammalian pre-implantation development Following fertilization of the oocyte, the resulting zygote undergoes several cell divisions to produce a small cluster of cells called the morula. The morula develops into the blastocyst, a ball of cells made up of an outer layer of trophectoderm, and a small cluster of cells called the inner cell mass. During normal development this process takes approximately 7 days to complete and can now be entirely reproduced using IVF technology. Pluripotent cells can be isolated at this point by removing

the inner cell mass from the blastocyst and continuing to culture these cells *in-vitro*. D = days post fertilization.

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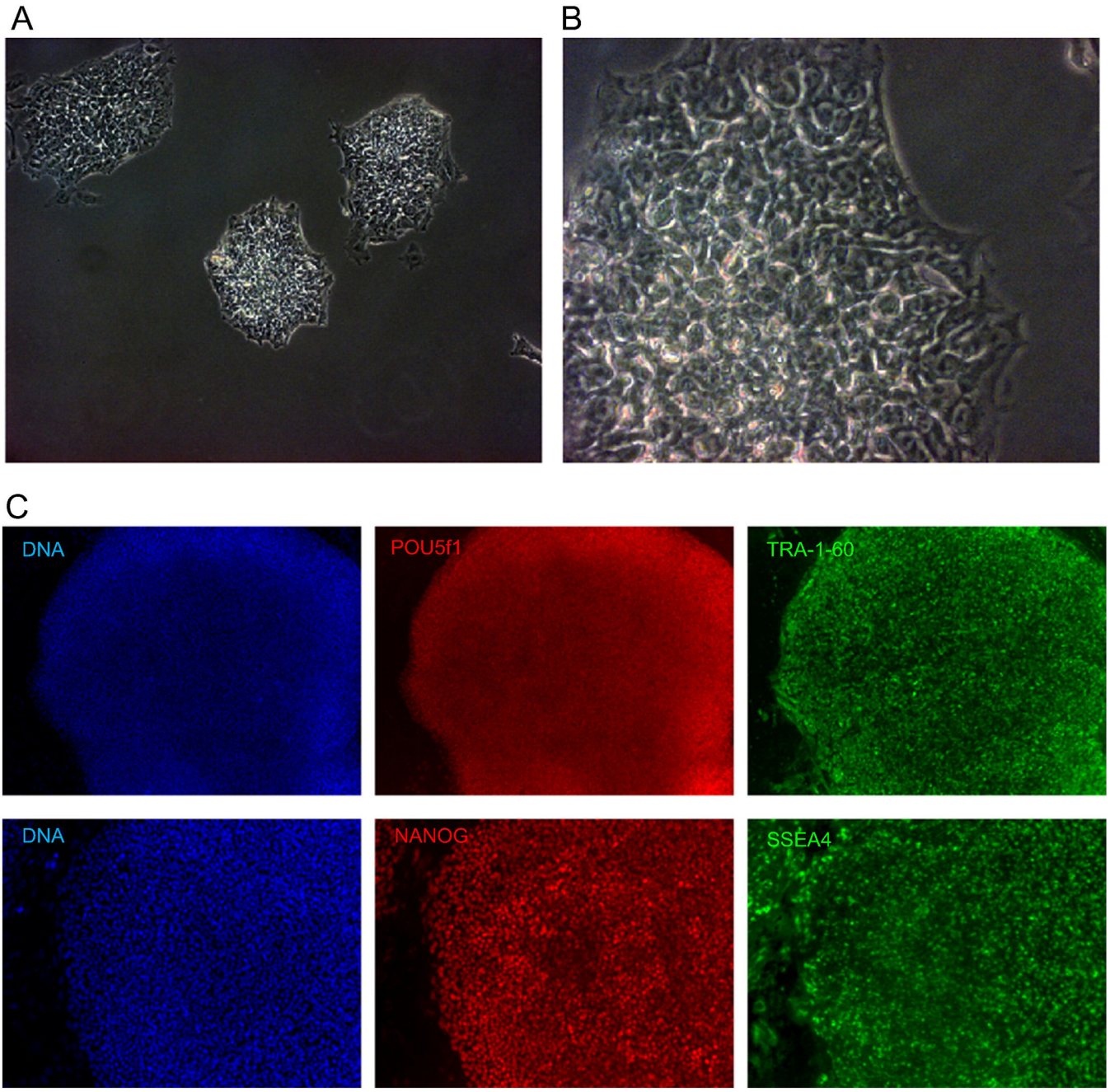

Fig. 3.

Characteristics of pluripotent cells. (A) Human pluripotent cells grown in feeder-free conditions show tight compact colony morphology with well defined colony borders containing cells with a high nucleus to cytoplasm ratio. (B) High magnification image of undifferentiated human pluripotent cells growing at high density with a high nucleas: cytoplasm ratio and prominent nucleoli. (C) Undifferentiated human pluripotent cells express the pluripotency markers POU5f1, TRA-1-60, NANOG, and SSEA4.

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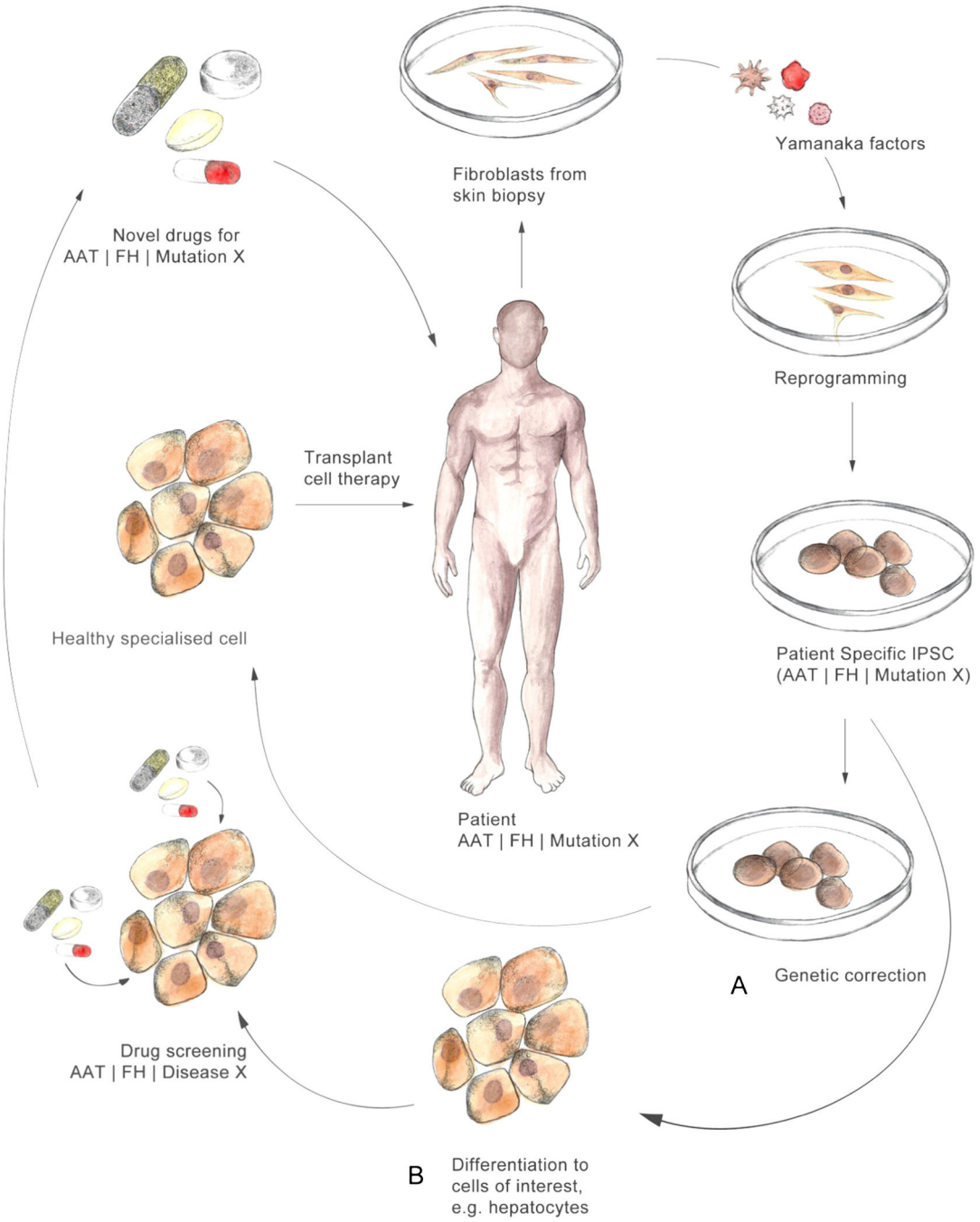

Fig. 4.

Applications of human induced pluripotent stem cells for regenerative medicine and drug screening. Induced pluripotent stem cells offer the opportunity to generate pluripotent cells from any genetic background starting with a small sample of somatic cells such as a skin biopsy. Cultured skin fibroblasts from patients with α-1-antitrypsin deficiency, familial hypercholesterolemia or “mutation X” can be reprogrammed using the Yamanaka factors to induced pluripotent stem cells. These hIPSCs may contain a mutation that can be (A) corrected using gene therapy and transplanted back into the patient or (B) differentiated into

a cell of choice for the purpose of screening novel pharmaceutical agents to correct the disease phenotype. Suitable drugs may then be identified as therapies to treat the specific disease or symptoms.

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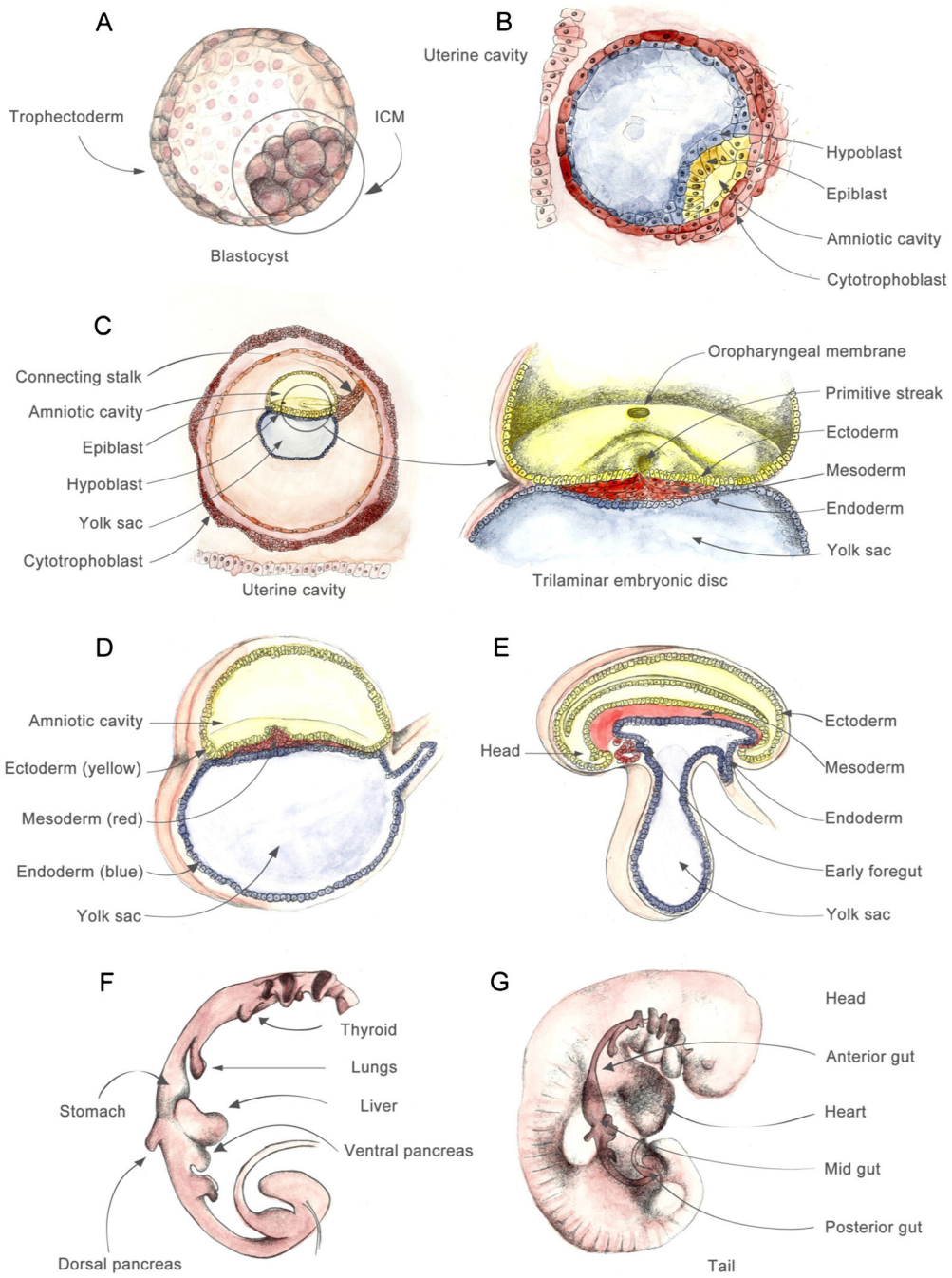

Fig. 5.

Mammalian gut development and organ specification. (A) The blastocyst is formed of an outer layer of trophectoderm that will form the placental structures and an inner cell mass that forms the embryo. (B) The ICM then flattens into a flattened disk made up of two cell layers the epiblast and the hypoblast. (C) Shortly after implantation gastrulation commences marked by the formation of the primitive streak, formed by the rearrangement and movement of cells in the epiblast that move through the primitive streak to produce the three embryonic germ layers. (D) As gastrulation continues the epiblast differentiates into the

three embryonic germ layers of ectoderm, mesoderm, and endoderm. (E) As development proceeds the endoderm layer expands forming a primitive gut tube with distinct foregut and hindgut domains. (E & F) The primitive gut tube becomes more specialized as different populations of cells along the gut tube form organs such as the lung, pancreas, and liver as well as the small and large intestines.

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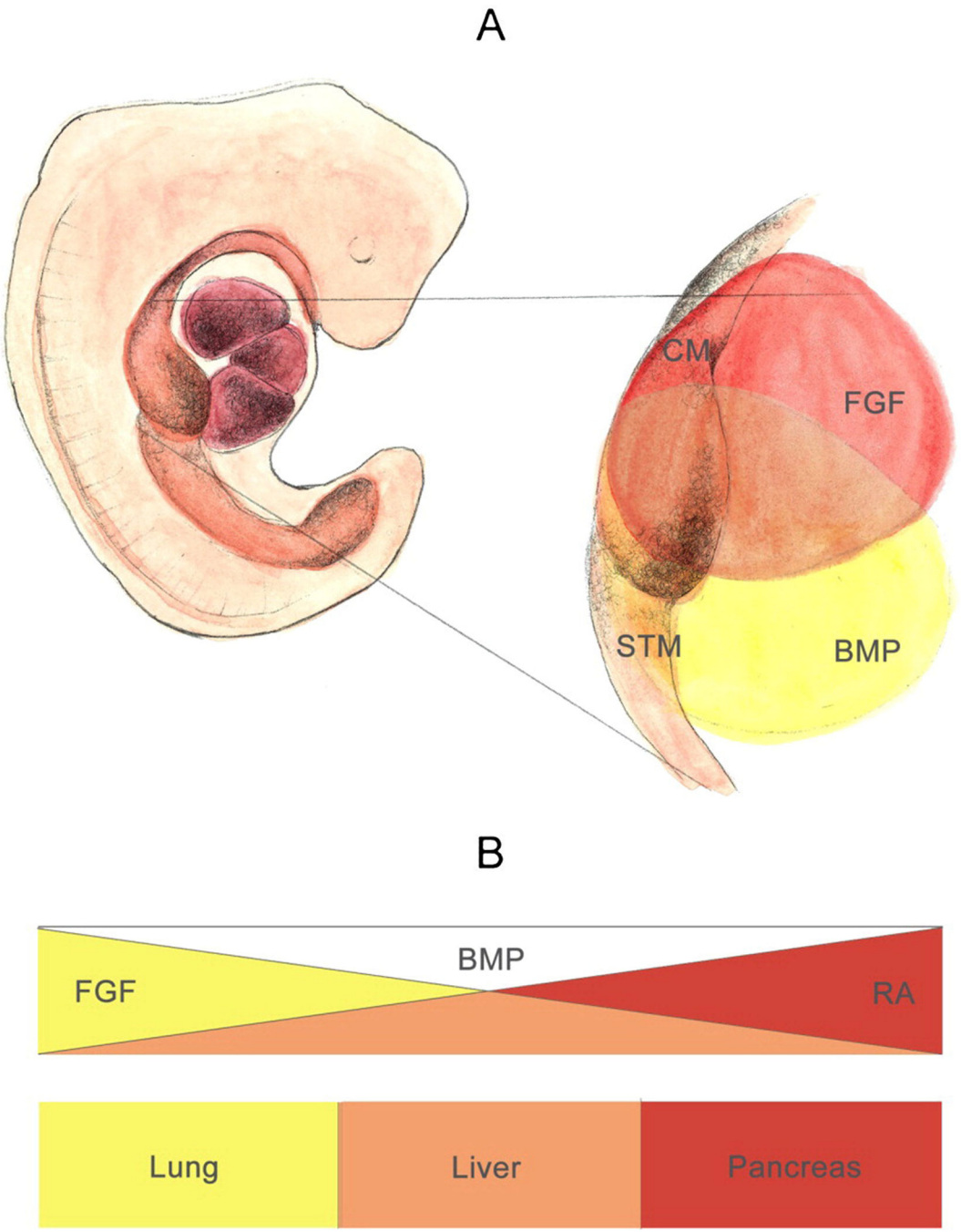

Fig. 6.

Patterning of the foregut by mesodermal tissues. (A) The domains of the foregut that give rise to diverse cell types such as those that form the lungs, liver, and pancreas are induced to become specialized cells in part by signaling from the overlying cardiac mesoderm and the septum transverse mesenchyme. (B) The foregut is patterned by a series of concentration gradients involving FGF, BMP, WNT, and RA signaling that induce precise expression of lineage specific genes.

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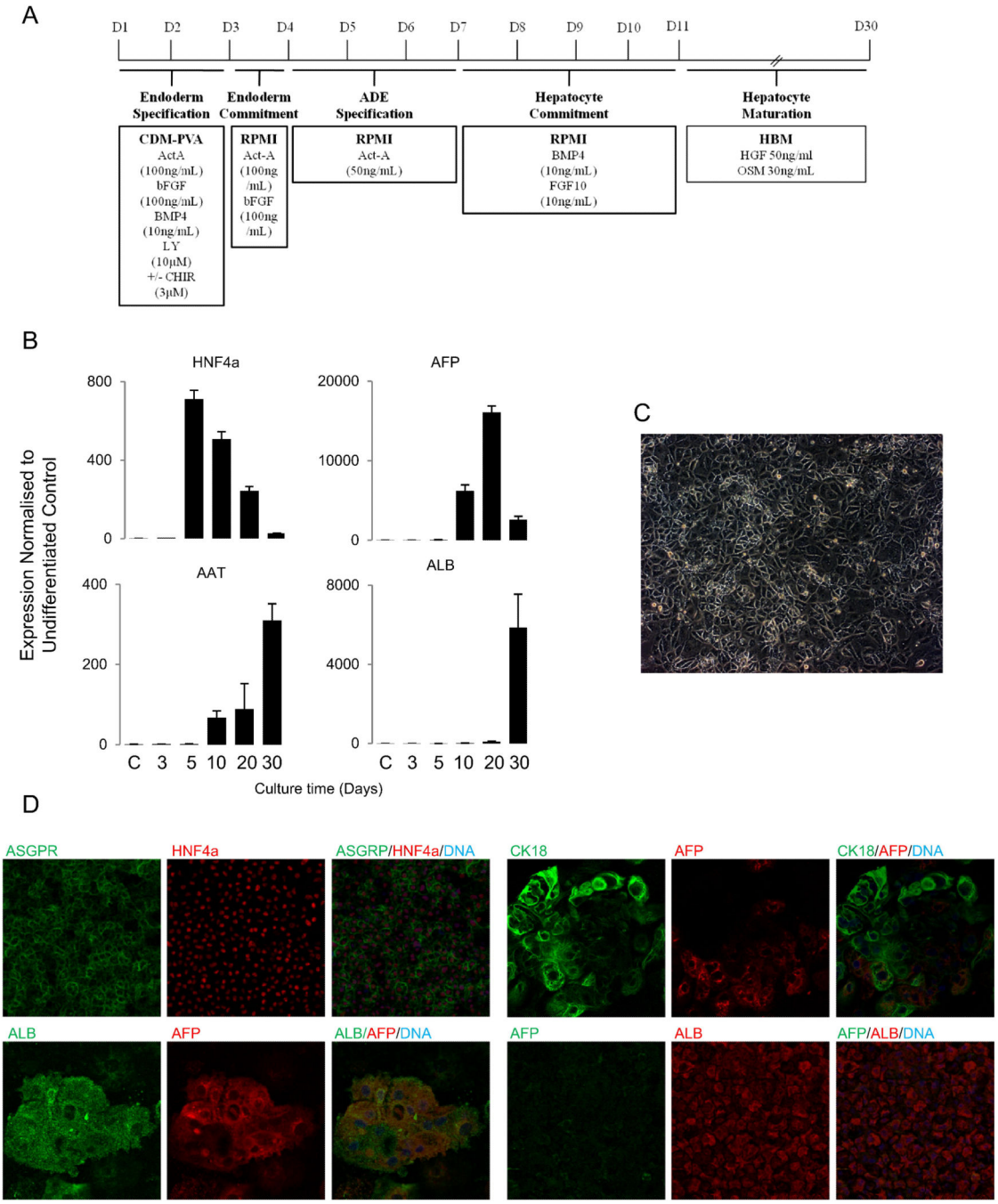

Fig. 7.

Protocol for differentiation of hepatocytes from hPSCs. (A) Protocol for producing hepatic cells using human pluripotent cells. (B) Gene expression profile of several hepatic markers showing early HNF4a expression followed by AFP, ALB, and AAT. The loss of AFP at D25 shows that at this point of the protocol cells are more adult like. (C) Morphological appearance of hepatic cells at D25 of the protocol. (D) Hepatocytes cultured for 25 days express several mature markers of adult human hepatocytes.

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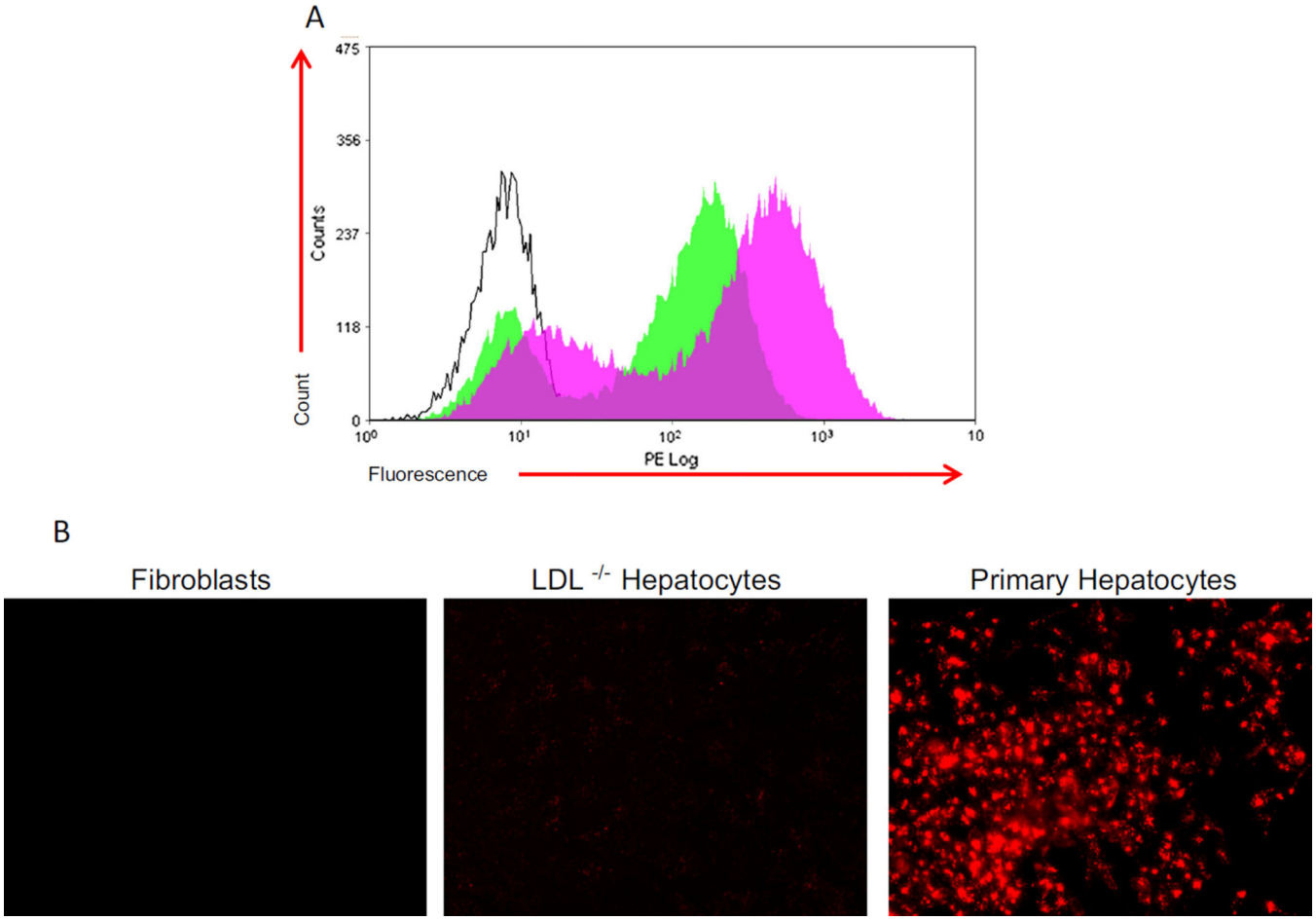

Fig. 8.

Characterization of FH hIPSC hepatocytes. (A) Flow cytometry showing LDL uptake from mouse embryonic fibroblasts (black line), compared to human primary hepatocytes (purple) and hepatocytes derived from patients with a mutation in the LDL receptor (green). (B) Fluorescent microscopy showing LDL uptake in mouse embryonic fibroblasts, hepatocytes derived from patients with a mutation in the LDL receptor, and human primary hepatocytes.

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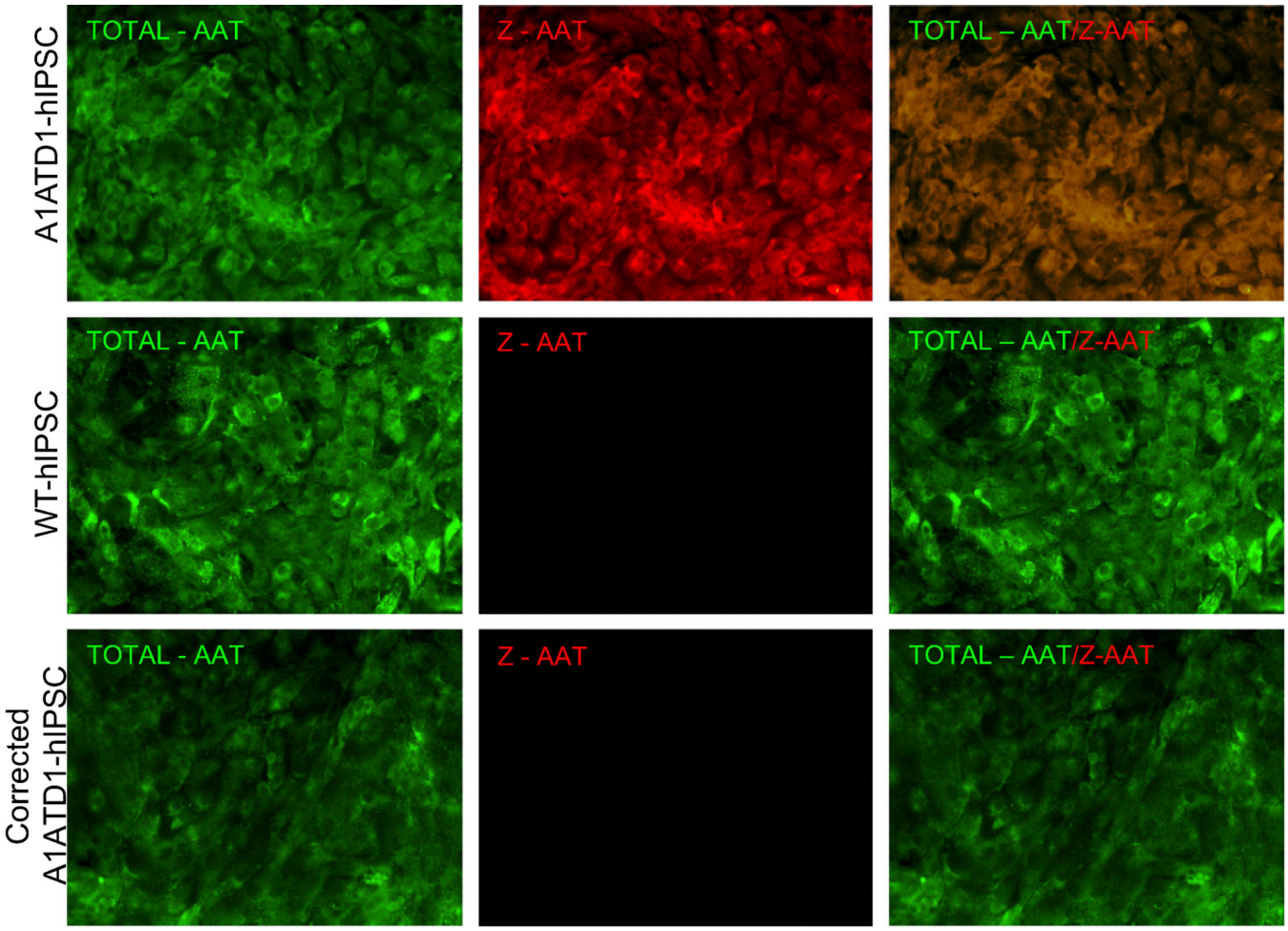

Fig. 9.

Characterization and genetic correction of α-1 -anti-trypsin deficient HIPSCs. Hepatocytes generated from either normal (WT-hIPSC) or α-1-anti-trypsin deficient HIPSCs (A1ATD-hIPSC) were stained with an antibody detecting either total AAT (green) or the misfolded polymeric AAT (red). A1ATD-hIPSC hepatocytes demonstrate accumulation of AAT polymers in the cytoplasm while WT-hIPSC stain negative. A genetically corrected version of the A1ATD-hIPSC line (corrected A1ATD-hIPSC) also shows an absence of the polymeric version of AAT.

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Table 1

List of human and mouse embryo carcinoma lines and their differentiation potential.

Cell line	Species	Pluripotent	Ectoderm	Endoderm	Mesoderm
F9	Mouse	N	N	N	N
P19	Mouse	Y	Y	Y	Y
PCC4	Mouse	Y	Y	Y	Y
PCC3/S640	Mouse	N	Y	Y	N
OTT650	Mouse	Y	Y	Y	Y
NTERA2	Human	Y	Y	Y	Y
NCCIT	Human	Y	Y	Y	Y
TERA2	Human	Y	Y	Y	Y
NCRG3	Human	Y	Y	Y	Y
NTERA2.cl.D1	Human	Y	Y	Y	Y
TERA2.cl.SP12	Human	Y	Y	Y	Y