

Effects of Influenza A Virus Infection on the Pluripotency and Proteome of Induced Pluripotent Stem Cells

By

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A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
In Partial Fulfilment of The Requirements of the Degree of

Master of Science

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ABSTRACT

Maternal influenza infection during pregnancy was reported multiple times as the possible cause of many defects and congenital anomalies. Apart from several cases of influenza-related miscarriage during various trimesters of pregnancy, some epidemiological data suggest a link between maternal influenza infection and genetic abnormalities in offspring. However, there are no reports yet describing cellular pathways that influence maternal influenza-induced congenital defects in the fetus. In the present study, using proteomic approaches, I utilized human induced pluripotent stem cells (hiPSCs) for modeling the intra-blastocyst infection with influenza virus to not only investigate the vulnerability and responses of pluripotent cells to this virus but also to determine the possible impacts of influenza on signaling pathways controlling embryogenesis. Immunoblotting and immunofluorescence microscopy indicated a considerable level of viral protein production in influenza A virus (IAV)-infected hiPSCs. However, IAV replication was restricted in these cells, but cell viability and pluripotency were negatively affected. These events occurred simultaneously with an excessive level of IAV-induced autophagy as well as cytopathic effects, suggesting the induction of spontaneous differentiation. Quantitative SOMAScan screening also indicated that minor changes in the proteome of hiPSCs corresponded to premature or abnormal spontaneous differentiation in these cells. Taken together, my results showed that IAV-modulated reduction in pluripotency of iPSCs is associated with significant activation of autophagy by this virus. Further investigations are required to explore the role of IAV-induced autophagy in leading pluripotent stem cells toward abnormal differentiation.

ACKNOWLEDGEMENTS

I owe my deepest gratitude to my advisor, Dr. Kevin M. Coombs, for all his continuous support and guidance, and for providing me with so much freedom to explore and discover different areas of my project. I express my warmest gratitude to my committee members Dr. Sanjiv Dhingra, Dr. Lorrie Kirshenbaum, Dr. Grant McClarty, and Dr. Darwyn Kobasa for their advices, innovative ideas, and insightful discussions during my meetings with them. Thank you also to Glen Sequiera for his generous technical support in preparing stem cell cultures. I would also like to thank Dr. John A. Wilkins and the members of the Manitoba centre for proteomics and system biology for providing an excellent research facility and exceptional lab training opportunity. My special thanks to past and present members of the Coombs' lab for their companionship and support. In no particular order, they are: Dr. Niaz Rahim, Protiti Khan, Mahamud Ur Rashid, Kathleen Glover, Affan Ali Sher, Hossaena Aylele, and Katherine Kresta. I am thankful to my colleagues in the department of medical microbiology for their precious comments. I am grateful to all positive energy sent from my friends Saeed, Hamid, Maryam, Sajjad, and Moin. Last but not least, I would like to thank my parents and siblings for giving me the motivation and support to finish this degree.

DEDICATION

To the four people who mean the world to me:
my parents, brother and sister.

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Abbreviations

A/PR/8/34 (PR8)	8 th isolate of influenza A strain from Puerto Rico in 1934
A549	Adenocarcinomic human alveolar basal epithelial cell
BSA	Bovine serum albumin
cRNA	Complementary RNA
DC	Dendritic cell
ESC	Embryonic stem cell
FBS	Fetal bovine serum
GO	Gene ontology
HA	Hemagglutinin
hiPSC	Human-induced pluripotent stem cells
hpi	Hours post infection
IAV	Influenza A Virus
IF	Immunofluorescence
IFN	Interferon
IL	Interleukin
IPA	Ingenuity pathway analysis
MDCK	Madin Darby canine kidney epithelial cell line
MOI	Multiplicity of infection
mRNA	Messenger RNA
mTOR	Mammalian/mechanistic target of rapamycin
NA	Neuraminidase
NANOG	Homeobox protein NANOG
NEP	Nuclear export protein
NES	Nuclear export signal
NK	Natural killer
NLS	Nuclear localization signal
NP	Nucleoprotein
NS1	Non-structural protein 1
OCT4	Octamer-binding transcription factor 4
PA	Polymerase acid protein
PANTHER	Protein analysis through evolutionary relationships
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PBS	Phosphate buffered saline
PFU	Plaque-forming unit
RdRp	RNA-dependent RNA polymerase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOMA	Slow off-rate modified aptamer
SOX2	SRY (sex determining region Y)-box 2
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRIM21	Tripartite motif containing 21
vRNA	Viral RNA
vRNP	Viral ribonucleoprotein

Chapter 1. Introduction

1.1. A Perspective on Influenza A Virus

A highly pathogenic disease known as fowl plague was reported as a pandemic in the early 1900s and resulted in high mortality rate among poultry. Later in the 1950s, an avian influenza virus was identified to be the causative agent of that epidemic [1]. During the first world war in 1918, a pandemic known as Spanish flu happened which resulted in the death of over 50 million people worldwide [2]. Mortalities were first assumed to be caused by bacterial pneumonia [3]. Later histological examinations of preserved tissues from victims identified viral genomes corresponded to avian H1N1 influenza virus [4]. In 1933, Influenza A virus (IAV) was initially isolated from the human population and observed to replicate productively *in vivo* [5]. Since then enormous attempts have been carried out in discovering the components of the virus and in revealing the clinical outcomes of an influenza virus infection. The influence of such findings expanded far beyond the influenza pathogenesis and not only have contributed to novel advances in immunology (e.g., interferon discovery) and elucidating protein structure but also extended our knowledge on general virology.

IAV, as a negative-sense single-stranded RNA and enveloped virus with a segmented genome, is classified as a member of the family *Orthomyxoviridae* which includes other types of influenza viruses like B and C as well as other viruses such as Thogotovirus, Isavirus, and Quarantivirus. Influenza viruses types A and B are known to cause frequent and occasional infections in humans, respectively. Based on antigenic characteristics of Hemagglutinin (HA) and Neuraminidase (NA) genes that code for surface glycoproteins, IAVs can be categorized into 18 HA and 11 NA subtypes [6, 7]. Although six NA subtypes

(N1, N2, N3, N6, N7, and N9) and six HA types (H1, H2, H3, H5, H7, and H9) have been found to result in human infections, pandemics in human populations were mostly seen to be related to 3 subtypes of HA (H1, H2, H3) and 2 subtypes of NA (N1, N2) [8]. Unlike the various subtypes of IAV, only two lineages were identified for influenza B viruses, including Victoria and Yamagata lineages [9]. Due to the importance of IAV in pandemics as well as its application in the present project, this type will be discussed more broadly hereafter.

The shorebirds and wild aquatic waterfowl are usually considered as the main natural hosts of IAVs [1, 10]. In addition to humans, these viruses can constantly adapt to a wide range of mammalian and avian species, such as birds, pigs, equids, and canines [1]. Depending on the host and viral genome, IAVs can be propagated either symptomatically or asymptotically. Over the past century, new and distinct human-adapted strains of IAVs have emerged to lead to several pandemics [11]. Because of the potential pandemic threats in the future, the significance of influenza research has become more obvious in recent decades. Regardless of several advances in the field, many questions yet remained to be investigated concerning the molecules involved in interspecies transmission, interactions of the virus with the host cell, modulation of signaling pathways during viral replication, and the immune responses against the virus. Given the possibility of another pandemic influenza outbreak, providing broad and detailed answers to such questions can promote our molecular understating of IAV pathogenesis for developing much more effective antiviral therapies and vaccines against the virus.

1.2. Influenza A Virus Structure and Proteins

The spherical virions of IAVs are 120 nm or less in diameter and obtain lipid membranes from the host cell, which create the envelope of the virus particle (Figure 1). These viruses are pleomorphic, as some filamentous particles with elongated shapes have also been observed and corresponded to IAVs [12]. The lipid bilayer membrane encompasses HA and NA surface glycoproteins that together form distinctive spikes with an approximate ratio of four to one, respectively [13]. The Matrix-2 (M2) proteins are incorporated in the lipid membrane and stick out from the surface, while the Matrix-1 (M1) protein is located below the envelope. The Nuclear Export Protein/Non-structural Protein 2 (NEP/NS2) is another structural part of the viral particle situated at the core [14]. Surrounded by the envelope membrane containing M1, M2, HA, and NA proteins, the inner viral core has helical superstructures constituting ribonucleoproteins (RNPs) made up of viral RNA segments, which are coated with Nucleoproteins (NP) and a single copy of a polymerase protein per RNP [15, 16]. Each IAV particle packages at least one copy of each of the eight unique RNA segments.

As the main surface antigenic protein and a virulence factor, HA plays a role in binding to the host cell receptors. The 2,6-linked sialic acid receptor is usually recognized by human IAV HA protein, while HA in avian IAV binds to 2,3-linked sialic acid [17]. In order to make HA fully functional and active, proteolytic cleavage is needed, which breaks this protein to HA1 and HA2 subunits [18]. HA also paves the way for the fusion of the virion with the host endosomal membrane. Due to the acidic pH of the endosome that induces a conformational change in HA, fusion pores are generated by HA2 peptide to pass

viral RNPs into the cytoplasm [17]. The adaptive immune system can recognize HA and produces neutralizing antibodies after replication of IAV [19].

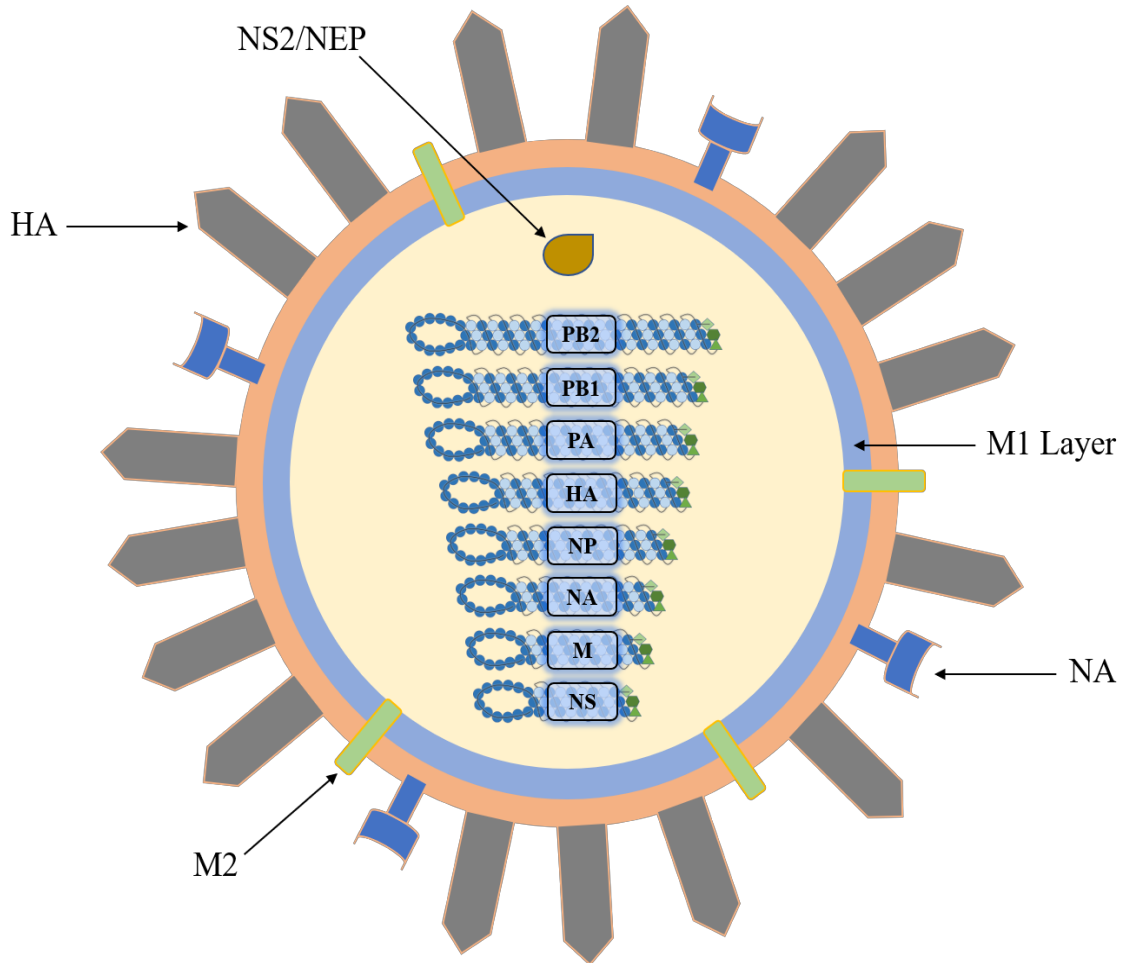


Figure 1. Influenza virion structure

A cross-sectional schematic diagram representing structural components of IAV. The lipid membrane encompasses hemagglutinin (HA), neuraminidase (NA) and proton ion channel protein M2. Matrix (M1) protein exists as a layer under the lipid membrane. Viral ribonucleoproteins (RNPs) are located in the inner section of the virion and possess negative sense RNAs encoding viral proteins.

NA is another surface antigen and an integral membrane glycoprotein in the structure of this virus. This protein is involved in releasing progeny viruses from infected cells. Although HA in newly produced viruses tends to bind with sialic acid receptors during the budding stage, residues of these receptors on the cell surface are cleaved by the viral NA not only to prevent recognition by the HA but also to allow the spread and release of progeny virions from one cell to another [18]. Most of the antibodies against the NA are found to be not neutralizing.

As the most abundant protein in IAV virion, M1 creates a bridge between inner core components and the surface proteins of the virus because of its location below the envelope and its connection with RNPs and cytoplasmic tails of membrane proteins [20]. M1 is assumed to participate in the assembly of progeny viruses by sequestering the viral components to the area of assembly at the plasma membrane. Moreover, together with NEP/NS2, M1 plays a role in conducting the nuclear export of viral RNPs [21]. M1 can be found in both cytoplasm and nucleus of infected host cells [1] and also has been reported to be essential for the budding process, as noted by its contribution and necessity in the generation of virus-like particles [22].

M2 is an integral membrane protein that acts as a proton ion channel through directing protons from the acidified endosomes into the virus, resulting in the separation of RNPs from the viral core because of low pH and their release into the cytoplasm for completing the uncoating step [23]. Its membrane-bound domain contributes to the assembly of viral proteins and RNPs via interaction with M1 [24].

RNA dependent RNA polymerase complex in IAV is a virulence factor comprised of basic polymerase protein 1 (PB1), basic polymerase protein 2 (PB2), and acidic polymerase

protein (PA). Cap snatching is a process by which small host RNA segments are exploited for starting viral transcription. PB2 is known to bind to the 5' cap of pre-messenger RNA (mRNA) molecules, and PA synthesizes mRNA cap from infected cells using its endonuclease function to trigger viral transcription [25-27]. PB1 plays important roles in maintaining structural integrity and catalytic activity of RNA polymerase [28]. The sequential addition of nucleotides is catalyzed by PB1 following cap snatching, leading to the elongation of viral mRNA. Another role of PB1 is initiation of transcription and replication, which is accomplished via binding the viral RNA (vRNA) and complementary RNA (cRNA) [29, 30]. PB1-F2 and PB1-N40 are two truncated proteins that are encoded by alternative open reading frames (ORF) in PB1 gene [31, 32]. As a virulence factor, PB1-F2 is capable of inducing apoptosis by interaction with host cell proteins [33].

NP can be highly expressed in IAV-infected cells and is noticeably abundant in the virion. It coats nearly the entire vRNA, thus considered to act as the structural part of RNP. Using the nuclear localization signal (NLS), NP transports vRNPs into the nucleus after uncoating to initiate transcription [34].

NEP, former known as NS2, recruits the export machinery and conducts the export of newly generated RNPs from the nucleus to the cytoplasm after transcription for the assembly of new virions. NEP/NS2 interacts with chromosomal region maintenance 1 (Crm1), the export receptor, and various nucleoporins to mediate the export of vRNPs [35]. The N-terminus of NEP/NS2 contains a methionine/leucine-rich nuclear export signal (NES), which is necessary for proper export of RNPs [36].

During the active life cycle of IAV, NS1 is expressed in considerable amounts in infected cells as a non-structural protein and usually taken into account as a marker for

successful replication. NS1 localization in nucleus and cytoplasm can be aided by the RNA binding domain of NS1 that contains NES [37]. The normal expression of NS1 in infected cells suppresses the interferon beta (IFN- β) promoter, causing less interferon (IFN) production [38]. Partial deletion of NS1 was demonstrated to attenuate IAV and its pathogenicity by allowing development of immune responses in both avian and mammalian species [39-41]. In addition to inhibiting host immune responses, IAV NS1 is known to regulate viral protein production, viral RNA synthesis, and mRNA splicing [42].

1.3. Influenza A Virus Life Cycle and Replication

The life cycle of IAV is initiated by the binding of HA protein to N-acetylneuraminic acid (sialic acid receptor) on the surface of targeted host cells (Figure 2). After attachment, the virus penetrates through the cell surface by receptor-mediated endocytosis and is taken into a cellular endosome. The conformational change in HA subunits that is induced by the low pH of endosome exposes the fusion peptide at the amino end of HA2, in order to fuse the viral envelope with the endosomal membrane, ultimately opens up pores that aid in transporting vRNPs into the cytoplasm of the cell [17, 43]. The uncoating process is further completed by M2 protein, which directs the influx of H⁺ ion from the endosome into the virion, leading to the separation and release of RNP complexes from M1 protein by interrupting protein-protein interactions [23].

RNPs of IAV need to enter the nucleus and rely on nuclear functions for transcription and replication of viral RNA [21]. Since influenza RNA synthesis happens in the nucleus and the transport of vRNPs depends on nuclear import machinery, all proteins present in RNP complexes like NP, PB1, PB2, and PA encompasses nuclear localization signal (NLS)

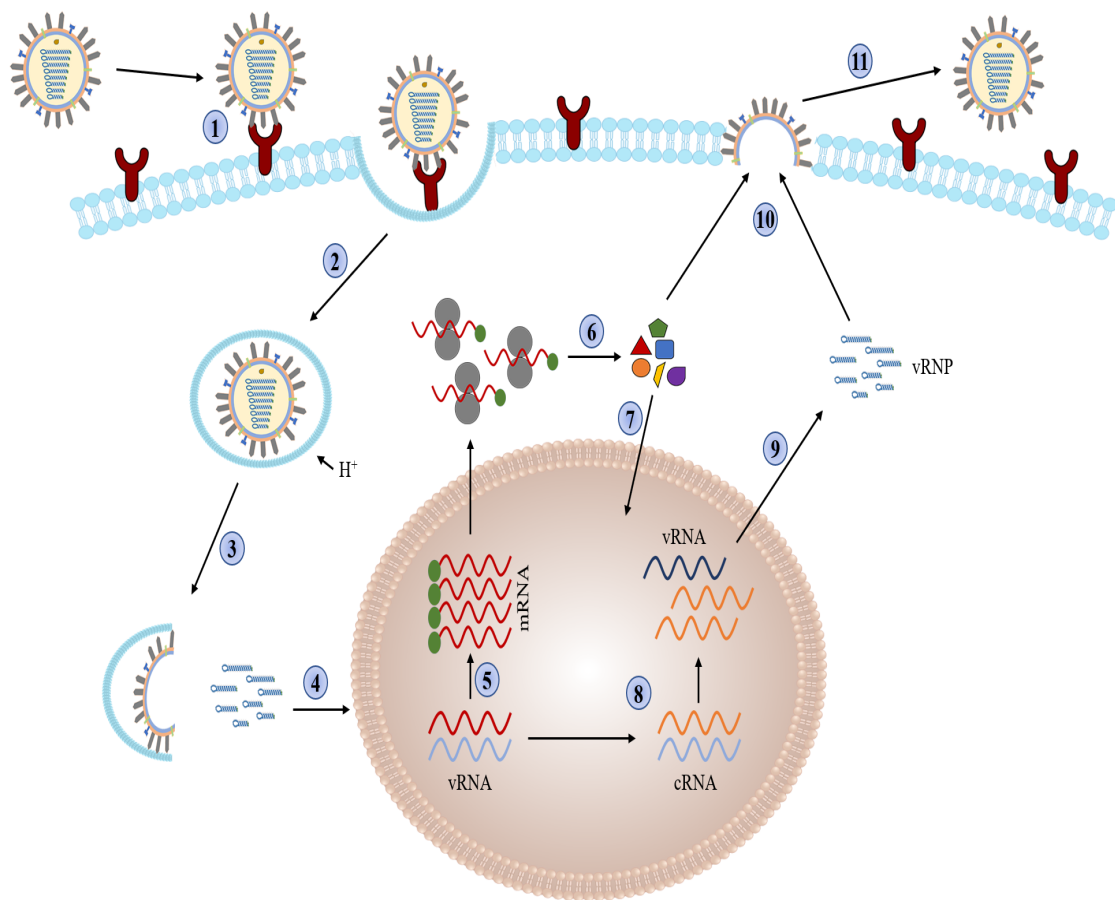


Figure 2. Influenza virus life cycle

(1) Virus attaches cell membrane via sialic acid receptor. (2) The receptor-mediated endocytosis facilitates viral entry. (3) Acidification of endosomal membranes results in the fusion between viral endosomal membranes. (4) Viral ribonucleoproteins (RNPs), which include viral RNA (vRNA), polymerase complex and nucleoproteins (NP), are released toward the nucleus. (5) Viral RNAs are utilized for transcribing viral mRNAs. (6) Translation of viral proteins occurs in the cytoplasm. (7) Certain viral proteins are carried back to the nucleus. (8) The synthesis of viral genomic RNA happens inside the nucleus. Apart from transcription, vRNAs are also produced in the nucleus for progeny viral

particles. Initially, exploiting negative sense RNA as a template, viral polymerase generates complementary positive sense RNA (cRNA). Afterward, using the template cRNA, viral polymerase creates more negative sense genomic vRNA. These newly synthesized vRNAs are then coated with NP and linked to polymerase complexes, ultimately resulting in the formation of vRNPs. (9) New vRNPs are exported to the cytoplasmic area. (10) Virions are assembled at cell membrane. (11) The budding of progeny particles is facilitated through neuraminidase activity.

to exploit the cellular nuclear import machinery [36, 44]. Despite the presence of NLSs on various components of RNPs, such signals on NP were determined to be adequate and critical for the import of vRNA [45].

After reaching the nucleus, the incoming negative-sense viral RNA transcribes into mRNA in a primer-dependent manner. As incomplete copies of the vRNA template, these mRNA products are required to be capped and polyadenylated within a two-step replication process. Unlike mature cellular mRNA that possesses both 5' methylated cap and poly (A) tail in the 3' end, IAV vRNP does not have a 5' cap and only contains a poly(A) tail. Because the 5' capped primer is needed for viral mRNA synthesis, the virus seizes cellular pre-mRNA transcripts for beginning its own transcription through the cap-snatching process, which is regulated by the cap-binding feature of the PB2 and endonuclease activity of the PA [46]. Following the binding of PB1 with the 5' end of the vRNA, the polymerase is allosterically altered to permit the binding of PB2 to 5' cap of the cellular pre-mRNAs [25, 47]. The endonuclease function of PA then promotes the cleavage of mRNA cap for viral transcription [48]. Benefiting from vRNA as a template, PB1 catalyzes the sequential addition of nucleotides to elongate viral mRNA chain. This elongation process goes on until uridine residues mediate signals for polyadenylation [46]. In contrast to cellular polyadenylation that employs certain poly (A) polymerase for producing the poly (A) tail on mRNA transcripts, the polyadenylation of influenza virus mRNAs is carried out by the transcription of vRNA-encoded consecutive stretch of uracil residues into positive sense adenosines [16]. Once the polyadenylation is completed, viral mRNAs are exported through the nuclear membrane for initiation of their translation. IAV is also known to take

advantage of host's splicing machinery for generating additional proteins in 7th and 8th segments of its viral RNA using alternative splicing mechanism [46, 49].

The synthesis of viral genomic RNA begins with the recognition of negative sense RNA as a template by RNA-dependent RNA polymerase (RdRp) complex, and cRNA is synthesized, which is a full-length, positive-sense copy of the vRNA. Then the generated cRNA is utilized by viral RdRp as a template to create more negative sense viral RNAs. Newly synthesized vRNAs are then coated with NP and linked to polymerase complexes, ultimately resulting in the formation of vRNPs [50]. The formation of nuclear pores by Crm1-mediated pathway is currently assumed to be the major route that IAV RNPs follow to be exported into the cytoplasm [36, 51]. A complex is proposed to be created by M1, NEP/NS2 and RNPs inside the nucleus that facilitates vRNP export. Based on this model, upon binding of M1 to the vRNP and NEP/NS2, interactions occur between NEP/NS2 and receptor Crm1 as well as various nucleoporins, eventually provoking the nuclear export of vRNPs into the cytoplasmic area [52].

All 8 RNA segments are required to incorporate into newly produced virions in order to keep the progeny particles in the infectious state. The exact mechanism for packaging viral RNA segments is not clearly characterized, but two various models have been considered. The first model suggests that the vRNA segments are incorporated randomly into budding virions. Studies have shown that more than eight vRNPs can be used by virions, which may prove the existence of a full complement of eight vRNPs [53, 54]. In contrast, the second model proposes that each vRNA segment contains unique signals to allow selective packaging of each segment into new virions [55]. These packaging signals exist within coding and non-coding regions at both the 5' and 3' ends of some vRNAs [51,

56, 57]. Such a segment-specific packaging model is assumed to happen through unique RNA-RNA or protein-RNA interactions [30]. In support of this model, studies have reported that specific deletion of RNA segments interferes exclusively with packaging of targeted wild-type RNA segment and packaging of other segments remains unaffected [57-60]. Most of reverse genetics findings confirm the selective packaging model.

To generate active and complete virions, all structural proteins and vRNPs are needed to be shifted to the plasma membrane of the infected cell. The surface glycoproteins HA and NA together with M2 are carried through trans-Golgi network towards the cytoplasmic membrane by using the exocytic pathway [61]. HA and NA are capable of attaching to the lipid rafts of the plasma membrane, but M2 does not depend on the attachment to such cellular microdomains [20]. vRNPs are transported by M1 to the location of viral assembly either through the cytoskeleton or by piggy-backing on cytoplasmic tails of viral surface glycoproteins NA and HA [61]. It is assumed that M1 abundance at the inner leaflet of the lipid bilayer induces the outward curvature of the plasma membrane, finally initiating the virion budding process [22]. A deformation in the plasma membrane is caused by HA and NA, and the polymerization, as well as the formation of the inner structure of new virions are started by M1 that is attached to the cytoplasmic tails of HA and NA [62]. M1 is proposed to recruit M2 in budding location for changing the membrane curve at the neck of the buds. This alteration leads to membrane scission and the separation of enveloped virions [62]. Once enveloped virions are detached from the plasma membrane, the HA of progeny particles binds to the sialic acid receptor on the surface of the cell, but this attachment is removed by the sialidase activity of NA to pave the way for the release of the virus [17, 18].

1.4. Influenza A Virus Interactions with Host Proteins

As intracellular parasites, IAVs need to extensively interact with cellular proteins and exploit host cell machineries for maintaining successful viral replication as well as evading host's immune responses. Many host cell proteins have been observed to be regulated differentially during influenza infection not only to aid IAV life cycle but also to modulate cellular stress response following infection [63]. Such differentially expressed proteins are mostly related to various pathways and biofunctions that control immune responses, signal transduction, transcription, and cell adhesion [64-67].

Using genome-wide RNAi screenings, Karlas et al. have discovered 287 human host factors that are required for the replication of influenza and appear to play roles in the essential functions of the early-to-middle phases of infection, including nuclear export of vRNA and viral protein synthesis [68]. Moreover, mass spectrometry analyses have found over 1,000 host proteins coprecipitate with IAV proteins that contributed to the development of the network of virus-host protein interactions based on the individual steps of the viral life cycle [69]. The interactions between different IAV proteins and cellular proteins depend on the stage of infection to result in a productive level of viral replication. In recent years, some other studies have also found that IAV NP and RNA-dependent RNA polymerase complex, comprising of PA, PB1, and PB2, interact with several host proteins for regulating viral life cycle, consequently disrupting other normal cellular processes like pre-mRNA splicing [70-72]. Despite that IAV NS1 protein is not packaged into new progeny particles, having multiple functional interactions with host proteins together with its high level of expression after successful transcription and translation indicate that the potential roles of this protein within active phases of viral replication may rely on

regulating different host cell proteins [42, 73, 74]. More than 400 cellular proteins were identified so far to interact with IAV NS1, based on ViroHostNet 2.0 online database. Apart from involvement in replication of IAV, these NS1-interacting host proteins are also contributing to the regulation of other biological processes such as apoptosis, autophagy, and host immune responses [42, 75]. Functional analysis of these virus-host interactions may provide valuable insight into potential preventive and therapeutic strategies that can be developed to target host proteins and associated mechanisms promoting IAV replication.

1.5. Host's Immune Responses Against Influenza A Virus

After entering respiratory epithelium and infecting epithelial cells, IAVs face the innate immune system as the first line of defense, which uses pathogen recognition receptors (PRRs) to detect vRNA as a pathogen-associated marker pattern (PAMP). Three types of PRRs that are capable of targeting IAVs include Toll-like receptors (TLR), retinoic acid-inducible gene I (RIG-I) and NOD-like receptor family LLR-pyrin domain containing 3 (NLRP3) [73, 76].

It is assumed that TLR3 triggers the production of pro-inflammatory cytokines through recognizing foreign RNA structures that can be present in phagocytosed virus-infected cells, thus restricting the viral replication [77]. IAV possess ssRNA, but TLR3 was reported to mostly target double-stranded RNA (dsRNA) in the endosome [78]. However, TLR7 is able to recognize the IAV ssRNA in the endosomes of plasmacytoid dendritic cells (DC), leading to the activation of either nuclear factor- κ B (NF- κ B) or interferon regulatory factor 7 (IRF7), which both can elevate the expression of type I IFN and pro-inflammatory

cytokines to suppress the formation of new progeny viruses and provoke antibody responses [76].

RIG-1 receptors are located in the cytoplasm and can recognize the 5' triphosphate of viral ssRNA after its release from the nucleus towards the cytoplasmic region [76, 79, 80]. Upon recognition of ssRNA, binding of RIG-1 with ATP causes conformational changes that enhance the binding of the mitochondrial antiviral signalling protein (MAVS) with RIG I caspase recruitment domains, subsequently mediating the activation of NF- κ B and IRF3 via producing pro-inflammatory cytokines and type I IFNs, respectively [81, 82].

The NLRPs are known to create inflammasome complexes that activate procaspase 1 and cleave pro-IL-18 and pro IL-1 β into their active structures [76]. It has been reported that NLRP3 activates an inflammasome after cellular stress and membrane damage which induces cytokine production via two signals [83, 84]. The first signal is elicited by TLR, Tumor Necrosis Factor Receptor (TNFR) and IL-1 receptor and triggers the transcription and translation of pro-IL18, pro-IL-1 β and NLRP3 genes through activating NF- κ B [76, 84]. The influenza-induced damages in the host cell provide the second signal, which promotes the activity of caspase 1 and secretions of IL-18 and IL-1 β [85, 86].

In addition to PPRs, immune cells like macrophages, natural killer (NK) cells, and dendritic cells (DCs) also play important roles in developing innate immunity against IAV infection. Alveolar macrophages can be activated after infection and eliminate IAV-infected cells by phagocytosis [87]. These activated macrophages can result in IAV-associated pathologies by producing nitric oxide synthase 2 (NOS2) and tumor necrosis factor alpha (TNF- α) [88, 89]. Furthermore, antigen-specific T cells are developed by such activated macrophages to regulate adaptive immune responses [90]. Using a process known

as antibody-dependent cell cytotoxicity (ADCC) for identifying distinct antibodies, NK cells are able to target and demolish IAV-infected cells [73]. During the attachment of the virus to the host cells, the IAV HA protein can be recognized by cytotoxic receptors of NK cells, leading to the destruction of infected cells [91, 92]. DCs act as antigen presenting cells (APCs) through presenting viral antigens to T lymphocytes, thereby linking innate and adaptive systems of immunity. After infection with IAV, DCs obtain and present antigens with major histocompatibility complex (MHC) by applying two different mechanisms. In the first mechanism, proteasomes in the cytosol of DCs infected with IAV can load viral proteins onto MHC class I molecules in the endoplasmic reticulum. Afterward, these MHC-I complexes that are loaded with viral peptides can be carried to the plasma membrane through the Golgi network and presented to virus-specific CD8+ cytotoxic T cells (CTLs) [73]. Based on the second mechanism, DCs acquire antigens via phagocytosing influenza viruses and their host cells that undergone apoptosis. After the degradation of viral proteins in the endosomes, resulting antigenic peptides can be loaded on MHC-II molecules, which are then shifted towards the cell membrane for presentation to CD4+ helper T cells [73].

Another antiviral mechanism against IAV infection is the elevated expression of IFN-stimulated genes (ISGs) in adjacent cells by Type I IFN response. Molecules such as MX proteins, IFN inducible transmembrane (IFITM) proteins, and Protein kinase R (PKR) are considered as some efficient products of ISGs against IAV infection. For example, cytosolic protein MXA is stimulated by IFN response and can constrain the transcription of IAV by interacting with PB2 and NP [93]. IFITM proteins restrict the attachment of IAV and endocytosis by inhibiting its fusion with the plasma membrane [76, 94, 95]. As a

serine/threonine kinase, PKR triggers eukaryotic translation and can limit the viral protein translation by binding dsRNA [76]. Despite the presence of ssRNA in influenza particles, secondary structures that are composed between vRNA termini can stimulate PKR activation [96]. Moreover, The upregulation of PKR after IAV infection evokes apoptosis and autophagy, leading to the restricted spread of progeny virions from the host cell [97]. On the other hand, due to high mutation rates, the segmented structure of vRNA, and selective pressure from the immune system, IAVs not only develop new strains by antigenic shift or drift to escape from adaptive immune system, but also bind viral proteins to different antiviral factors for avoiding innate immune responses [98].

IAV NS1, as a multifunctional protein, evades innate immune responses by interacting with the host proteome using various mechanisms. For instance, RIG-I signalling cascade can be inhibited by three different mechanisms controlled by NS1. In the first mechanism, NS1 represses the activity of NF- κ B and IRF3, which are needed for IFN transcription [38, 99, 100]. The second mechanism aids NS1 for binding to TRIM25 to hinder IFN production [101, 102]. Another mechanism stimulates the interaction of NS1 with a subunit of the cleavage and polyadenylation specificity factor (CPSF30), an essential molecule for converting pre-mRNAs into mature mRNAs of antivirals and IFNs [103]. NS1 can also bind to PKR to block its antiviral function by limiting its conformational changes [99]. The activity of PKR is regulated through a complex that P58^{IPK} forms with heat shock protein 40 (HSP 40). Using an unknown mechanism, IAV upregulates the expression of P58^{IPK} and detaches this cellular inhibitor from the regulatory complex of PKR [74, 104]. Also, IAV M2 protein interacts with P58^{IPK}-HSP40 complex and restricts P58^{IPK} release, ultimately resulting in decreased levels of protein synthesis, promoted apoptosis, and

release of viral particle [105]. Regardless of NS1, other IAV proteins can also participate in escaping from host innate immune responses. For example, type I IFN production can be inhibited by the binding of IAV PB1-F2 to MAVS, and PB2 is capable of suppressing IFN- β production through binding and restricting the activity of IFN promoter stimulator 1 (IPS-1) [106, 107].

1.6. Anti-influenza Drugs

M2 inhibitors and NA inhibitors are considered the main classes of clinically approved antiviral drugs against IAV. Amantadine and Rimantidine are M2 inhibitors that affect the proper uncoating of vRNPs at initial stages of infection by targeting M2 ion channel protein [108]. These inhibitors were found to be inefficient against Influenza B viruses. Besides, M2 inhibitors resistant-strains have made obstacles in the efficiency of these drugs for hindering the spread of IAVs in human population [109]. NA inhibitors like Zanamivir and oseltamivir block the release of progeny virions by targeting IAV NA protein. These inhibitors can be effective against various strains and found to control some outbreaks efficiently [110]. Nevertheless, new strains have emerged recently that are resistant to NA inhibitors as well [111, 112]. Ribavirin is another antiviral that inhibits the replication of both influenza A and B viruses in humans. However, because of problems like toxicity and inefficient oral delivery, this drug is administrated limitedly and required to be delivered through intravenous injection or by inhalation [113]. According to *in vitro* and *in vivo* studies, viramidine, a ribavirin prodrug, can effectively suppress the replication of IAV and is associated with fewer toxic effects than ribavirin [114]. Favipiravir, a nucleoside analog, targets the viral RNA-dependent RNA polymerase and is found to be effective in treating

infections with many negative-strand and positive-strand RNA viruses [115]. It has shown promise against both seasonal and avian influenza viruses *in vitro* and *in vivo* and no resistant mutants have yet been reported either in animal models or in patients [115-117]. Xofluza (Baloxavir marboxil) is an endonuclease inhibitor and another antiviral medication against influenza A and influenza B viruses. It is taken orally in a single dose and suppresses the cap-dependent endonuclease function of the influenza polymerase, resulting in the inhibition of cap snatching process [118, 119].

1.7. Teratogenic Effects of Influenza Infection

Over the past several decades, epidemiological data from multiple influenza pandemics and seasonal epidemics have shown a remarkably high rate of fatalities in pregnant women and the fetus, suggesting that gestation puts both mother and offspring at higher risk of complications triggered by severe influenza infection [120]. For example, during the 2009 pandemic season in the United States, less than nine percent of influenza-infected pregnant women died and twelve percent of all pregnancy-related deaths were caused by influenza infection [121]. However, despite the lack of proper non-pregnant control groups, pregnancy-related mortality rate was found to be considerably high during the pandemics of 1918 and 1957, ranging from 20 to 45% of influenza-infected pregnant women [122-124]. Pregnancy-related physiologic changes for maintaining embryogenesis and tolerating the fetus as a foreign allogenic tissue, consecutive phenotypic and functional alterations of different immune cells such as NK cells and T lymphocytes, and the effect of hormonal imbalance on the function of CD4⁺ T helper cells, are assumed to be only a

part of the factors that lead to defective immune responses against influenza in pregnant women [125-127].

Apart from cases confirming influenza-induced miscarriages or stillbirths during various trimesters, maternal influenza infection in pregnancy was reported multiple times as the possible cause of some developmental defects and congenital anomalies [128]. For instance, cleft lip, neural tube defects, and cardiac malformations were seen more often in newborns of mothers who experienced seasonal influenza infection during pregnancy [129]. According to population-based studies, the risk of giving birth to an infant with a right-sided obstructive heart anomaly, particularly tricuspid atresia, can be elevated by maternal influenza at the periconceptional stage of pregnancy [130]. Moreover, meta-analyses found that some types of cardiac deformities, such as aortic ventricular septal defect and valve atresia/stenosis, and other congenital abnormalities like hydrocephaly, digestive system defects, limb reduction anomalies, and cleft lip, have higher risk to emerge after maternal exposure to influenza during the first trimester, compared to other various sorts of fetal malformations (Figure 3) [131-134].

While some studies link influenza infection to fetal abnormalities, the molecular mechanisms behind such teratogenic effects in human influenza infection have remained elusive. A few studies have suggested that toxic metabolites derived from influenza-induced fever and hyperthermia are behind the manifestation of congenital anomalies in offspring rather than viral particles themselves [135]. Hyperthermia itself may contribute to vascular malformations, as observed in animal embryos [136]. The elevated body temperature in mother has already proved to induce abnormal cell death during the

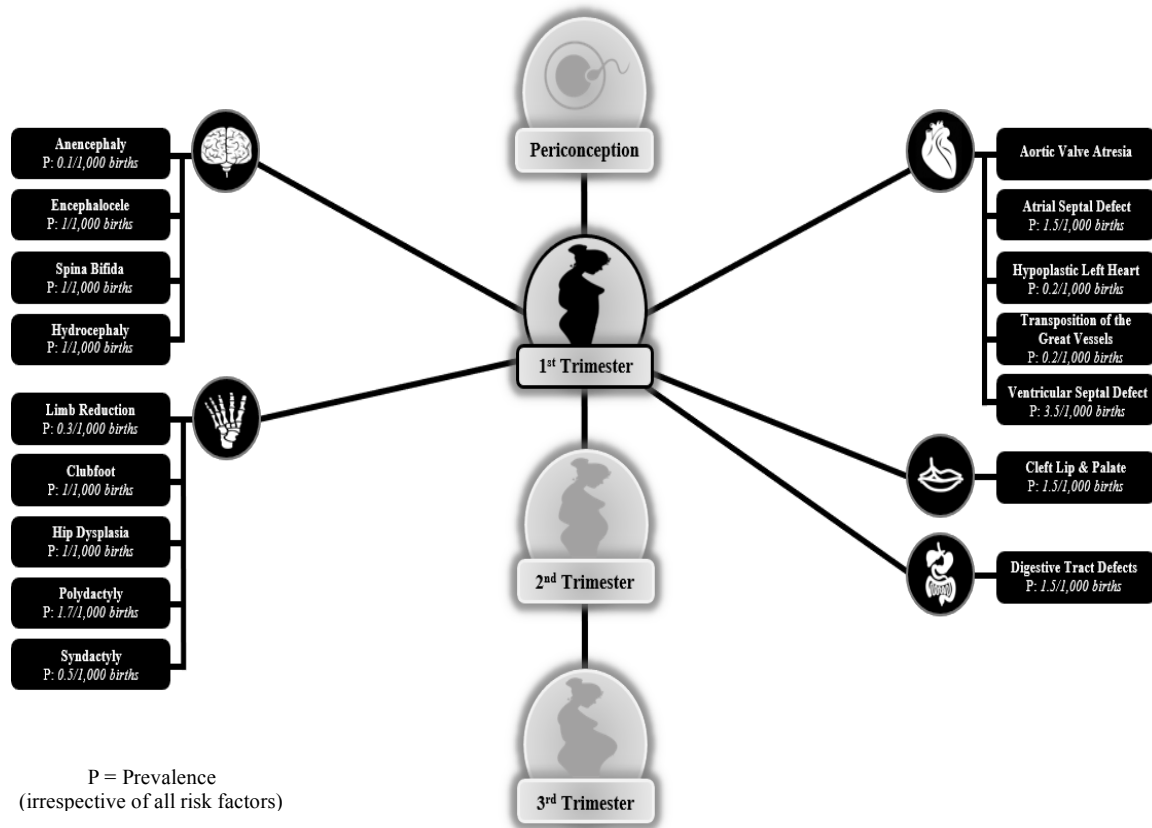


Figure 3. Congenital anomalies with high risk of incidence after maternal exposure to influenza infection during first trimester of pregnancy

Meta-analysis showed statistically significant relationships between first trimester influenza exposure and a large number of congenital malformations, such as heart defects, brain/central nervous system/spinal cord anomalies, orofacial deformities, digestive system malformations, and limb reduction (Luteijn et al., 2014).

embryonic development in various species [137, 138]. Likewise, apart from virus-triggered physiological changes, influenza virus can induce apoptosis both *in vitro* and *in vivo* for its own benefit and release of more progeny particles [139-141]. For example, during cardiogenesis, apoptosis acts as an essential mechanism for remodeling outflow tract

myocardium to appropriately link ventricular chambers with suitable arterial trunks [142]. Given that cardiogenesis initiates from the first trimester of pregnancy, maternal flu-associated fever, hyperthermia, influenza particles alone or combination of all during this course of gestation may cause abnormal apoptosis in developing cells [143]. Besides, the influence of influenza is not limited to induction of cell death and may subvert host cell proteome, causing dysregulations in various signaling pathways controlling organogenesis.

A few post-mortem histological analyses in pregnant women infected with influenza virus subtypes H3N2, H5N1, or H1N1, have not only confirmed viremia and extrapulmonary spread of the virus in a variety of maternal tissues, but also the transmission to fetal lung cells, amniotic fluid, fetal cardiomyocytes, histiocytes, cytotrophoblast cells, and fetal liver macrophages (Figure 4) [144-147]. Given the importance of the villous surface area for carrying oxygen and nutrient, it was also reported that maternal and fetal inflammatory interactions after placental infection with influenza might decrease the surface, and consequently may constrain fetal growth [146]. Although the transplacental passage of this virus is currently being debated and seems to occur rarely or might happen indistinctly, the differentiation and normal cellular functions of embryonic cells could be changed through minor feto-maternal viral interference in signaling pathways that regulate embryonic development.

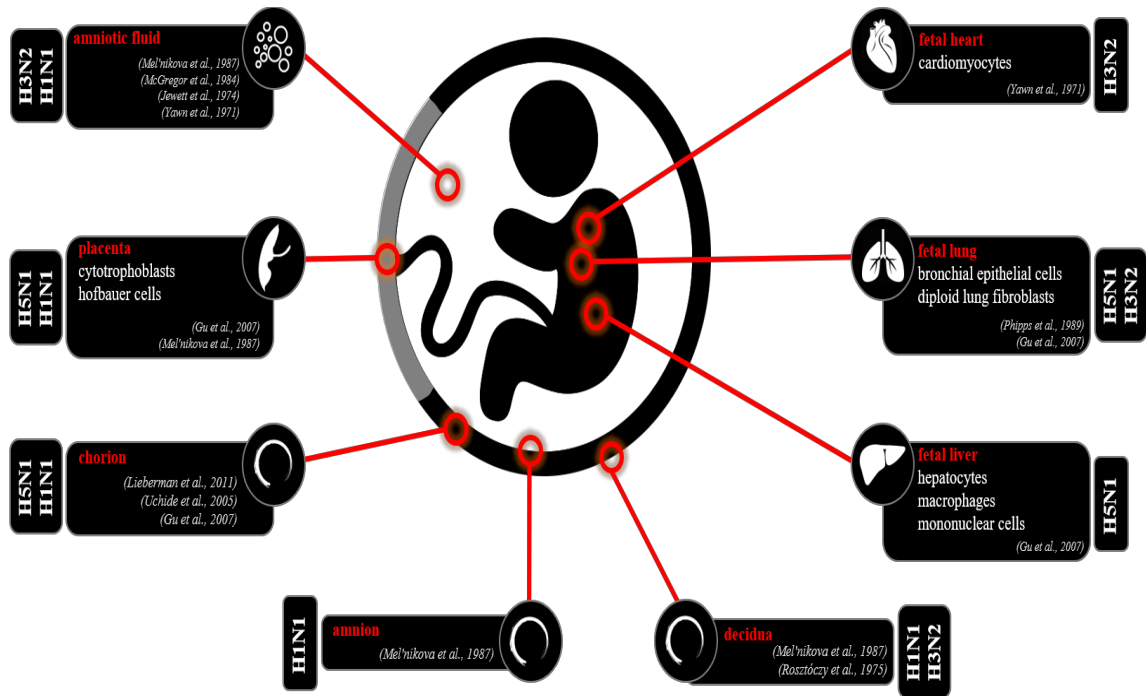


Figure 4. Cases confirming the transplacental passage of influenza virus

Although different studies reported the identification of viral sequences and antigens for three IAV subtypes from different fetal and placental tissues, only a few tissues were found with severe viral-induced cytopathological. The impacts of maternal influenza on the fetus are remained unclear. Viremia is assumed to happen occasionally, and thus transplacental passage seems to be rare or may occur indistinctly.

1.8. Effects of Influenza Infection on Stem Cells

In addition to some cases confirming the possibility of placental or intrauterine influenza infection, this virus replicates productively in certain multipotent or unipotent stem cell populations, including umbilical cord epithelial stem cells, lung epithelial stem cells, and neural stem cells [148-150]. For instance, Pringproa et. al demonstrated that infecting human neural progenitor cells differentiated from human embryonic stem cells with highly pathogenic avian H5N1 influenza virus triggers the secretion of inflammatory cytokines, such as IFN- α 2, IFN- β , IFN- γ , and IL-6, which may ultimately result in increased levels of inflammation and abnormalities in the central nervous system [150]. Nevertheless, Influenza A virus has been found to have a restricted replication and protein production in mouse embryonic stem cells (mESCs), but the viability of these cells was reduced even at lower multiplicity of infection (MOI), which indicates the relative vulnerability of the blastocyst's inner cell mass (ICM) to IAV [151]. ESCs are pluripotent cell populations that present only transiently in the blastocyst stage of embryonic development and give rise to epiblast and hypoblast, which ultimately result in the differentiation into germ layers during the gastrulation phase to initiate the formation of tissues and organogenesis. Pluripotency is an essential attribute of pluripotent stem cells (PSCs) and represents the stemness, differentiation capacity, and self-renewal ability of such cells. Since some of the influenza-modulated congenital defects are believed to originate from the first trimester of pregnancy when the unevolved and highly vulnerable, virus-sensitive placenta still develops, pluripotent stem cells must be taken into account to study the impact of influenza on early embryogenesis. Unlike differentiated cells, due to limited responses to most pathogens, pluripotent stem cells are recognized to lack an

interferon-based antiviral mechanism and an intrinsic antiviral route, showing that these cells evoke an underdeveloped innate immunity against viruses [152].

1.9. Gaps in knowledge

Interactions of influenza viral proteins with themselves and with host cell proteins are known to elicit substantial changes in the proteome, as noticed by several virus-induced impairments in host cellular machinery like affecting apoptosis and autophagy, activation of host immune responses and evasion from them, and decreasing host protein synthesis to expand viral protein production [42, 65, 75, 153-156]. None of these influenza-mediated alterations in the host proteome have been reported before on a proteomic scale in pluripotent cells. Additionally, cell death and survival signaling pathways in pluripotent stem cells, especially after different viral infections, are poorly understood [157]. Modifications in pluripotency, self-renewal, and germ layer differentiation pathways, as the most critical characteristics of pluripotent stem cells, have not yet been elucidated under infections with the vast majority of viruses, including influenza.

1.10. Rational and Goal

In recent decades, due to the increased prevalence of mortality from birth defects, many efforts have been made to maintain the health of infants both during embryonic stages and after birth in order to have a healthy human population more than before. Efficient methods and tools have been provided so far which can detect many anatomical, cytogenetic and genetic abnormalities in the fetus. Nevertheless, infants with congenital birth anomalies are still considered amongst the main issues in different societies. Influenza infection in

pregnancy has been reported many times as the cause of some defects and congenital anomalies. Several cases of miscarriage within various trimesters of pregnancy or birth with congenital defects suggest a relationship between influenza infection in pregnant women and abnormal development of fetal tissues. Some challenges like segmented genome of the virus, high mutation rate, and antiviral-resistant strains put both mother and fetus at higher risk of encountering IAV-induced complications. Thus, further studies are needed for developing more effective immunization strategies during pregnancy.

Many types of congenital defects are believed to emerge from the initial days of embryogenesis when a pregnant woman is more susceptible to IAV infection because of several physiological, hemodynamic, and immunologic alternations for tolerating the embryo. Moreover, considering the possibility of extrapulmonary passage, the placenta is not fully developed during the early stages of embryogenesis, thus cannot completely prevent the potential transmission of IAV into embryonic tissues. Therefore, modeling the early pregnancy may provide valuable information about the potential role of IAV infection in impaired embryogenesis, which can subsequently promote fetal immunization by targeting influenza-modulated mechanisms. Human induced pluripotent stem cells (hiPSCs) are derived through overexpressing pluripotency genes in somatic cells and can be used as a model for studying the effects of influenza virus not only on the pre-implantation stage of human embryonic development but also on the signaling pathways involved in the differentiation towards germ layers.

The overall goal of my project is to model the intra-blastocyst infection with IAV using hiPSCs to investigate the vulnerability and responses of these pluripotent cells to this virus,

and to determine the possible impacts of IAV on pluripotency and signaling pathways controlling stem cell differentiation.

1.11. Hypothesis and Objectives

I hypothesize that influenza A virus directly disrupts the pluripotency of hiPSCs, subsequently leading to dysregulations in signaling pathways that control the normal differentiation.

The objectives of my study are as follows:

Objective 1: to investigate the replication and protein production of IAV in hiPSCs

Objective 2: to determine the impact of IAV on the pluripotency of hiPSCs

Objective 3: to characterize the cellular vulnerability in hiPSCs after IAV infection

Objective 4: to screen the proteome of IAV-infected hiPSCs for identifying potential dysregulations in pathways controlling the initial differentiation signals

Chapter 2. Materials and Methods

2.1. Media

Madin-Darby canine kidney (MDCK) cells were grown in 1x complete Dulbecco's Modified Eagle Medium (DMEM; Gibco, cat# 12100-061), supplemented with 5% fetal bovine serum (FBS; Gibco, cat# 12483), 2 mM l-glutamine (Gibco, cat# 25030-081), 2 mM sodium pyruvate (100 mM; Gibco, cat# 11360-070) and 1x MEM non-essential amino acids (Gibco, cat# 11140-050). The medium for culturing human alveolar basal epithelial (A549) cells was composed of 1x complete DMEM, supplemented with 10% FBS, 2 mM l-glutamine, 2 mM sodium pyruvate and 1x MEM non-essential amino acids. Human induced pluripotent stem cells (hiPSCs) were maintained in TeSR™-E8™ basal medium (StemCell Technologies, cat# 05990).

In IAV infection experiments, A549 and MDCK cells were overlaid with serum-free medium containing 1x DMEM, supplemented with 2 mM l-glutamine, 2 mM sodium pyruvate and 1x MEM non-essential amino acids, while TeSR™-E8™ medium was used for infecting hiPSCs. To do plaque assay, MDCK cells were overlaid with 0.8% Avicel in FBS-free 1× DMEM media containing 2 mM l-glutamine, 2 mM sodium pyruvate, and 1x MEM non-essential amino acids. In both infection conditions for all cell types and plaque assay in MDCKs, overlay medium was further supplemented with 2.5 µg/mL trypsin, 1x gentamicin and 1x amphotericin B. Other than commercially obtained TeSR™-E8™ medium, all media and solutions were prepared in double distilled water (ddH₂O).

2.2. Cells and Viruses

iPS cells were generated from human peripheral blood mononuclear cells (PBMCs) using Sendai virus kit (Life Technologies) for forced expression of pluripotency genes, as described previously [158], then grown as colonies in E8 medium to reach the semi-confluent condition. MDCK and A549 cells were passaged in complete DMEM medium supplemented with 5% and 10% FBS, respectively, as mentioned in the previous section. Human influenza virus strain A/PR/8/34 (H1N1; PR8), a mouse-adapted strain, was amplified in MDCK cells by infecting at MOI of 0.01 for 48 hrs and concentrated at 64,000 $\times$ g for 2 hrs at 4 °C. Viral pellet was kept in the mixture of gel saline and glycerol for 24 hrs at 4 °C. After resuspension on the following day, virus was then aliquoted at -80 °C. The virus stock was then titrated by plaque assay on MDCK cells.

2.3. Infection and Plaque Assay

After washing semi-confluent hiPSC colonies 2 $\times$ with 1 $\times$ phosphate buffered saline (PBS; 137 mM NaCl, 0.3 mM KCl, 0.8 mM Na₂HPO₄, 0.1 mM KH₂PO₄), cells were infected with PR8 virus diluted in E8 medium to achieve different multiplicities of infection (MOIs), including 0.1, 1, and 5 plaque forming units (PFU)/cell. To compare IAV growth kinetics in hiPSCs with other influenza-permissive cell lines, A549 and MDCK cells also were infected at the same MOIs by diluting the PR8 virus in gel saline (137 mM NaCl, 0.2 mM CaCl₂, 0.8 mM MgCl₂, 19 mM HCO₃, 0.1 mM Na₂B₄O₇, 0.3% (w/v) gelatin). An equivalent number of cells were mock-infected using either only E8 medium for pluripotent stem cells or gel saline for other cells. At 12 and 24 hpi, infected and mock-infected hiPS and A549 cells were harvested for immunoblotting.

To quantify the virus' yield by plaque assay, supernatants were collected from all three cell types at assigned time points and serially diluted 1:10 in gel saline. Diluted supernatants then were added to sub-confluent monolayers of MDCK cells plated in six-well dishes. Following an hour adsorption, cells were overlaid with 0.8% Avicel in FBS-free 1× DMEM media containing 2mM l-glutamine, 2mM sodium pyruvate, and 1x MEM non-essential amino acids, and supplemented with 2.5 µg/mL trypsin, 1x gentamicin and 1x amphotericin B. After 72 hrs incubation at 35°C to permit plaque formation, cells were fixed with 2% formaldehyde for 30 min and then stained with crystal violet for 1 hr. Viral titer was calculated as plaque forming units (pfu)/mL by counting plaques 4 hrs after washing stained cell monolayers [64], based on the following formula:

$$\text{PFU/mL} = \frac{\text{Average Number of Plaques}}{\text{Dilution Factor} \times \text{Volume (mL) of diluted virus added in monolayer}}$$

2.4. Immunoblotting

At time points 12 and 24 hpi, mock- and influenza-infected hiPS and A549 cells were scraped into cold PBS, then pelleted at 500 × g for 6 min, and lysed for 15 min in mammalian protein extraction reagent (M-PER™, Thermo Scientific, cat# 78501) supplemented with HALT™ protease inhibitor (Thermo Scientific, cat# 78440). After clearing cell lysates by centrifugation at 14,000 ×g for 15 min, supernatant protein contents were collected, and the Pierce™ BCA Protein Assay Kit (Thermo Scientific, cat# 23227) was applied to measure protein concentrations.

Equal amounts of proteins were loaded per lane into SDS-polyacrylamide gels (SDS-PAGE), fractionated, and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were blocked with 5% skim milk in TBS (Tris-

Buffered Saline) buffer containing 0.1% Tween 20 for 2 hrs, and then incubated overnight with the desired primary antibodies at 4°C. Following overnight incubation with primary antibodies, membranes were probed with either rabbit or mouse HRP-conjugated secondary antibody (all primary and secondary antibodies are listed in Table 1) for 1 hr at room temperature, and the bands were visualized through enhanced chemiluminescence (ECL) detection machine (Amersham-Pharmacia Biotech). ImageJ software was used to quantify virus-to-mock ratios from the intensity of visualized bands. The quality of blots was optimized for contrast and brightness using image settings plugin of Microsoft PowerPoint.

2.5. Analysis of Cellular Morphology

To examine PR8 virus-induced cytopathic effect (CPE) development, infected and mock-infected hiPSCs were assessed by inverted microscopy (Nikon TE-2000) at intended MOIs and photographed using a Canon A700 camera. All micrographs were exported into PowerPoint for minimal adjustment of brightness and contrast. The analysis of stem cell colony mass and size was done through crystal violet staining in a 12-well plate. After washing 3 times with PBS, iPS cells were fixed with 4% paraformaldehyde for 15 min and then stained with 0.5% crystal violet solution in 4% paraformaldehyde for 10 min. The stained plate was washed twice with tap water, and infected colonies were evaluated and compared to mock-infected wells on the next day.

Table 1. List of primary and secondary antibodies used in this study

<i>Primary Antibodies</i>					
Antibody	Host	Clone	Catalogue	Assay	Source
Monoclonal α - NP	Mouse	F26-9		WB, IF	In-house
Monoclonal α - M1	Mouse	GA2B	MA1-80736	WB, IF	Thermo Fisher
Monoclonal α - NS1	Mouse	4E10		WB, IF	In-house
Monoclonal α - beta-actin	Mouse		3700	WB	Cell Signalling
Monoclonal α - CASPASE3	Mouse	3G2	9668	WB	Cell Signalling
Monoclonal α - CASPASE7	Mouse	C7	9494	WB	Cell Signalling
Polyclonal α - CASPASE9	Rabbit		9502	WB	Cell Signalling
Polyclonal α - BAX	Rabbit		2774	WB	Cell Signalling
Polyclonal α - BCL2	Rabbit		2872	WB	Cell Signalling
Monoclonal α - P53	Mouse	DO-7	48818	WB	Cell Signalling
Polyclonal α - LC3	Rabbit		L8918	WB	Invitrogen
Polyclonal α - P62	Rabbit		5114	WB	Invitrogen
Polyclonal α - ATG5	Rabbit		PA5-29694	WB	Invitrogen
Monoclonal α - NANOG	Rabbit	D7364	4903	IF	Cell Signalling
Monoclonal α - SOX2	Rabbit	D6D9	3579	IF	Cell Signalling
Monoclonal α - OCT4A	Rabbit	C30A3	9656	IF	Cell Signalling
Polyclonal α - TF	Rabbit		abx101259	WB	Abbeexa
Polyclonal α - SPARC	Rabbit		5420	WB	Cell Signalling
Monoclonal α - GAPDH	Rabbit	14C10	2118	WB	Cell Signalling
Monoclonal α - STAT3	Mouse	124H6	9139	WB	Cell Signalling
Polyclonal α - PSMA2	Rabbit		2455	WB	Cell Signalling
<i>Secondary Antibodies</i>					
Antibody	Host	Clone	Catalogue	Assay	Source
Polyclonal α - mouse HRP	Mouse		7076	WB	Cell Signalling
Polyclonal α - rabbit HRP	Rabbit		7074	WB	Cell Signalling
Alexa Fluor® 546 α - mouse	Goat		A11003	IF	Life Technologies
Alexa Fluor® 488 α - rabbit	Goat		A11034	IF	Life Technologies

WB: Western Blot, IF: Immunofluorescence

2.6. Assessment of Cell Viability

The trypan blue exclusion assay was used to determine cell viability. Briefly, PR8-infected or mock-infected hiPSCs were harvested at various post-infection time points by brief trypsinization to break up the colonies. Single cells derived from disaggregated colonies then were washed, stained with trypan blue solution and placed on a hemocytometer for counting. The percentage of viable cells was calculated based on the number of dead (blue) cells divided by the total number of cells counted, multiplied by 100. The cytotoxicity of autophagy drugs (Rapamycin and Bafilomycin A1) was determined by WST-1 cell proliferation assay (Roche, Germany) according to the manufacturer's instructions. In brief, hiPSCs or A549s grown in 96- well plates were treated with WST- 1 reagent for 90 min. at 37°C. Colorimetric changes were measured, and cell viability then was calculated compared with controls.

2.7. Indirect IF microscopy

For IF staining, iPS cells were grown to 60% confluence on chamber slides and then infected or mock-infected with the PR8 virus at MOI = 5. After fixing with 4% paraformaldehyde for 15 min at 12 and 24 hpi, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and blocked with 3% BSA blocking solution for 90 min. Afterward, cells were incubated overnight 4 °C with primary anti-NANOG, -Sox2, -Oct-4A (all three from Cell Singling), -NS1, -NP, and -M1 (all three made in-house) antibodies diluted in 1% BSA and PBS. After overnight incubation, cells were washed 5× with PBT buffer (PBS with 0.2% Tween-20) and treated for 1 hr with Alexa Fluor™ 488 and 546 (Invitrogen) secondary antibodies. Slides were then mounted using DAPI-Prolong® Gold

Antifade for nuclear staining, sealed using nail polish and preserved at 4 °C for imaging on the following day. Fluorescent images were acquired at 20× and 40× objective by Zeiss Axio Observer Z1 inverted microscope and further optimized and saved with AxioVision 4.8.2 software. ImageJ software was used to quantify the levels of fluorescence signal intensities.

2.9. Inhibition and activation of autophagy

Autophagic activity was blocked using autophagy inhibitor Bafilomycin A1 (Cayman Chemical). I utilized Rapamycin (Sigma-Aldrich) for promoting the level of autophagy. Both drugs were used in four different concentrations of 4000, 500, 50, and 5 nM for cytotoxicity analyses. Based on cell viability results, lower concentrations were selected for evaluating viral growth, autophagy, and pluripotency. In influenza-infected conditions, cells were first adsorbed with the virus at MOI of 5.0 for 1 hr and then treated with selected concentration of Bafilomycin A1 (5 nM) or Rapamycin (50 nM) mixed in cell-specific culture medium.

2.9. SOMAScan Screening

After measuring protein concentrations in three biological replicates by BCA assay, cell lysates were adjusted to 200µg/ml and sent for proteomic screening using an in-house SOMAScan assay platform (version 1.3), which uses modified nucleotides with high affinity known as SOMAmers (Slow Off-rate Modified Aptamers) (Figure 5). In total, the expressions of 1,307 proteins were determined from lysates isolated at 12 and 24 hpi from

mock-infected and infected hiPSCs at MOI of 5. Raw specific protein abundance values were obtained as relative fluorescence units (RFU).

2.10. Statistical and Bioinformatic Analysis

Data were collected in triplicate from all experiments. For growth curves, cell viability, and band intensity analyses, statistical differences were assessed by one-way or two-way ANOVA using Graph Pad Prism 6.0 and results were reported as means $\pm$ SDE. P-values < 0.05 were considered statistically significant. To analyze SOMAScan results, RFU raw expression values were imported into Microsoft Excel and converted to Log_2 for calculating fold-changes. P-values were determined using the two-tailed Student's t-test. To compare various biological replicates, z-scores were calculated from protein ratios within each replicate, as previously described [64, 159]. Briefly, all fold-changes not deemed to be significant by T-test were examined by Z-score, expressing each value as its number of standard deviations away from the population mean. Protein IDs from the whole examined proteome were uploaded to and analyzed by Ingenuity Pathway Analysis software (IPA). Panther databases were utilized to classify all significantly expressed proteins in gene ontology categories. Venn diagrams and heatmap were plotted using InteractiVenn and MORPHEUS (developed by Broad Institute, Cambridge, MA, USA) free online software, respectively. Western blot data were tested for significance by one-way ANOVA, using a significance cut-off of 0.05.

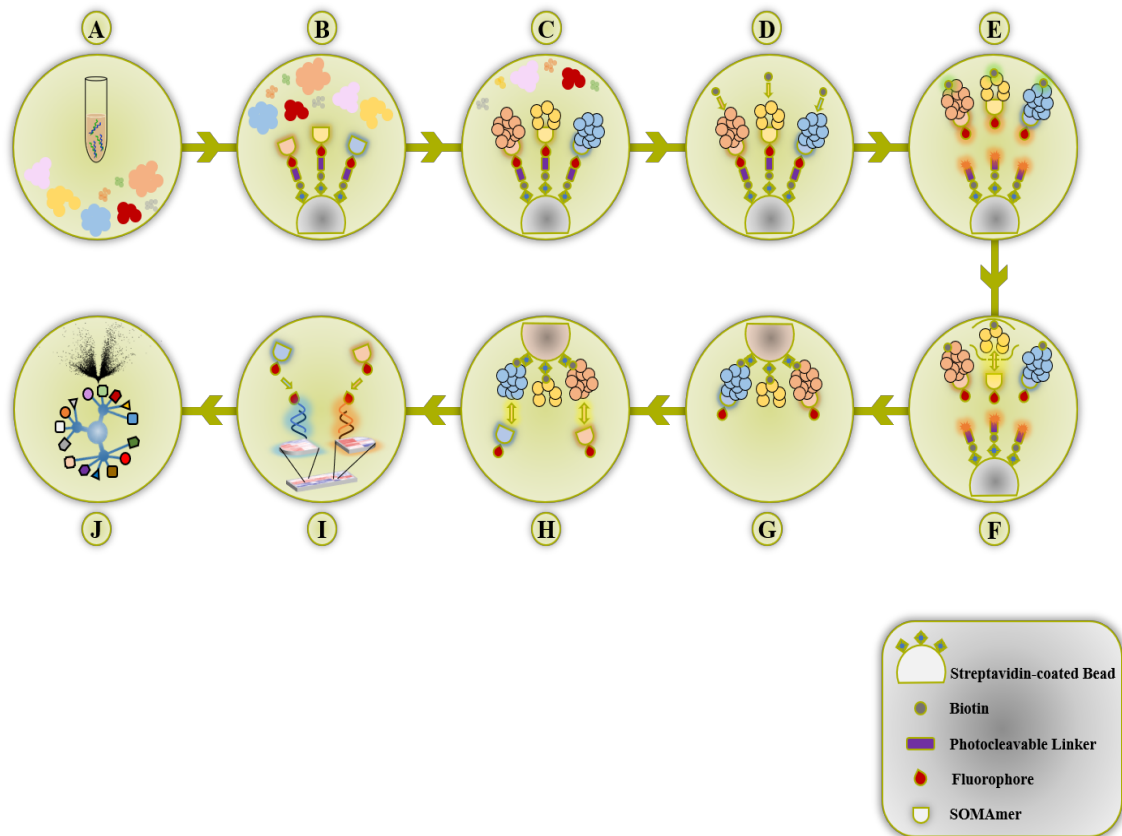


Figure 5: SOMAscan Workflow

After extracting cell lysate (A), a labeling complex consisted of a fluorophore, photocleavable linker, and biotin, connects SOMAmers to streptavidin-coated beads, and resulting chains are incubated with the mixture of proteins (B). Proteins then are attached to SOMAmers and establish noncognate (middle) and cognate (left and right) complexes on the beads (C). Afterward, beads are washed to detach unbound proteins, and biotin is added to tag proteins (D). The UV light reaction with photocleavable linker results in the release of SOMAmer-protein complexes from the beads (E) and isolated complexes are then incubated with a polyanionic competitor buffer, which selectively captures and removes nonspecific and non-complexed SOMAmers (F). Other sets of streptavidin-coated beads retrieve SOMAmer-protein complexes by biotin-tagged proteins (G). Using

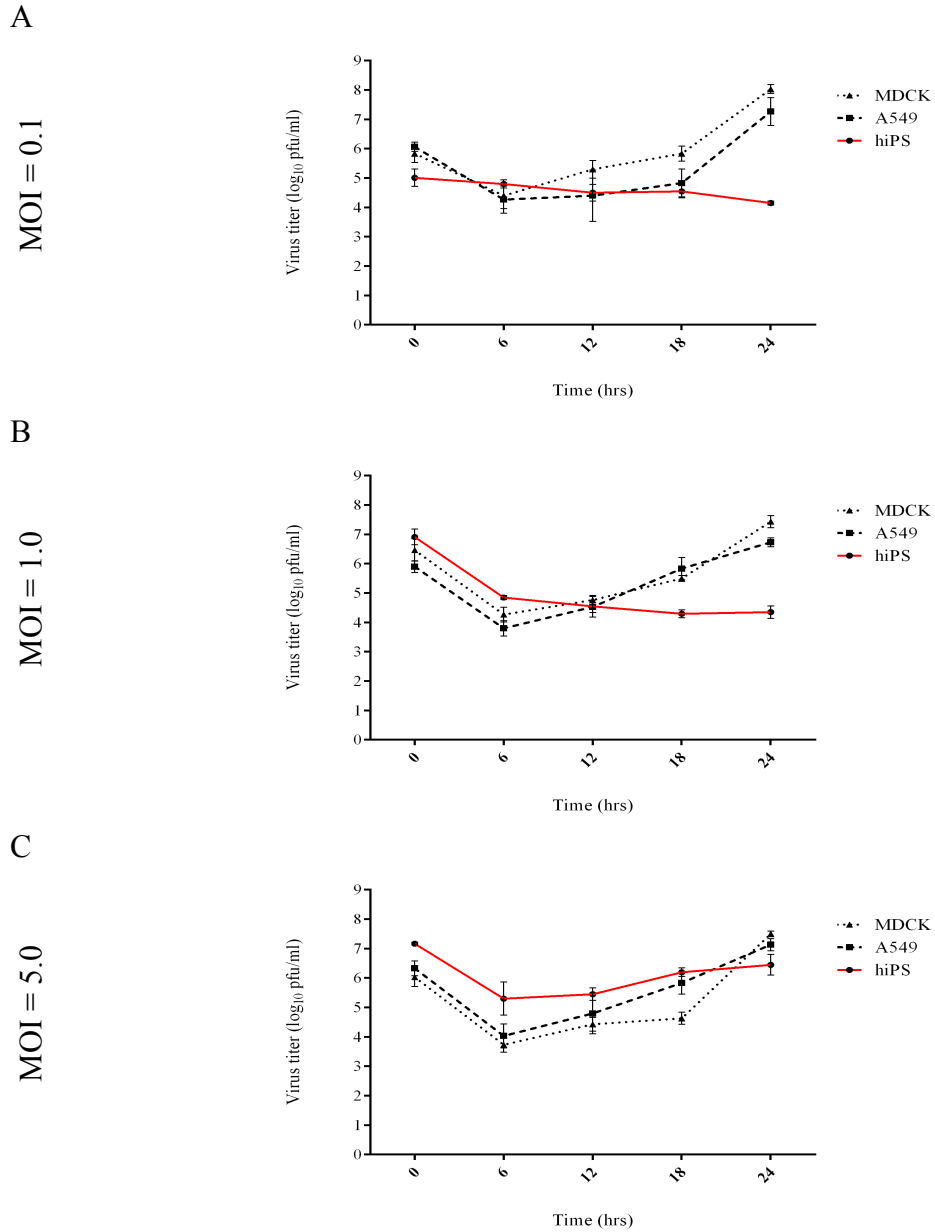
denaturing buffers, SOMAmers are separated from second beads (H) and undergo hybridization into complementary nucleotide sequences by microarray (I). After quantification based on fluorescence intensity, various types of bioinformatic analysis is performed on the protein expression profiles (J).

Chapter 3. Results

3.1. Influenza A/PR/8/34 replicates restrictively in hiPSCs

Human induced pluripotent stem cells are not routinely used for influenza studies; therefore, I first optimized growth conditions and the spread of influenza A/PR/8/34 (H1N1; PR8) within hiPSC colonies. Three multiplicities of infection (MOIs; 0.1, 1.0, and 5.0) were considered to evaluate the level of infectious progeny viruses secreted into the culture media over time by plaque assay. At the MOI of 0.1, viral growth was entirely restricted in hiPSCs, as no titer amplification was observed by 24 hpi, while I measured efficient titers of 10^8 and 10^7 PFU/mL at the same time point in MDCK and A549 cells, respectively, which are known to be permissive to influenza virus (Figure 6A). Unlike epithelial cells that continued producing progeny viruses at higher MOIs, similar restricted growth pattern was seen in hiPSCs at MOI of 1.0, and the viral titer only fluctuated slightly between 10^4 to 10^5 PFU/mL at various post-infection time points (Figure 6B). At 24 hpi, the maximum titer of 10^6 PFU/mL at MOI of 5.0 was attained, which appeared to be upward and just a little lower than the initial titer at 0 hpi (Figure 6C). However, I did not observe significant changes in the level of progeny viruses recovered from hiPSC culture supernatant at later time points (e.g., 36 or 48 hpi).

Influenza replication was further analyzed by Western blotting to determine the production of viral matrix 1 protein (M1), nucleoprotein (NP), and non-structural-1 protein (NS1). Despite no detectable expression at lower MOIs, viral proteins were clearly expressed in hiPSCs by 12 hpi at MOI of 5 and decreased a bit in abundance by 24 hpi (Figure 7A). Moreover, immunofluorescence microscopy not only documented the entry of virus and significant expression of viral proteins but also revealed that an MOI of 5.0



Published in Cell Death & Disease (Zahedi-Amiri et al. 2019) [160]

Figure 6. Restricted replication of influenza A/PR/8/34 in hiPSCs. A-C) Comparison of PR8 virus growth kinetics between pluripotent stem cells and IAV-permissive cell lines at different MOIs. hiPS, MDCK, and A549 cells were infected at MOIs of 0.1 (**A**), 1.0 (**B**), and 5.0 (**C**) to track and compare viral yield. Supernatants were collected and titrated for measuring viral growth by standard plaque assay at various time points.

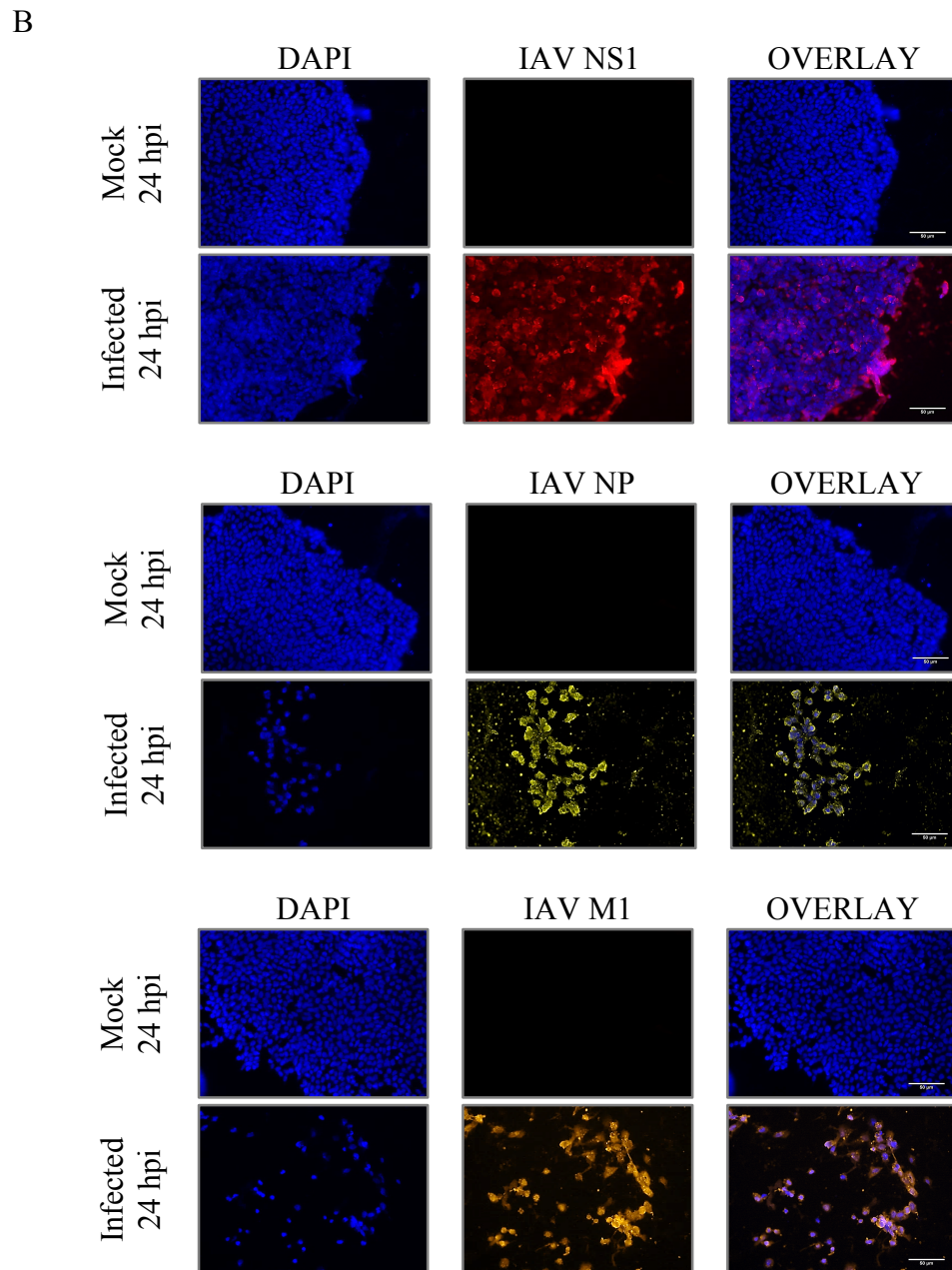
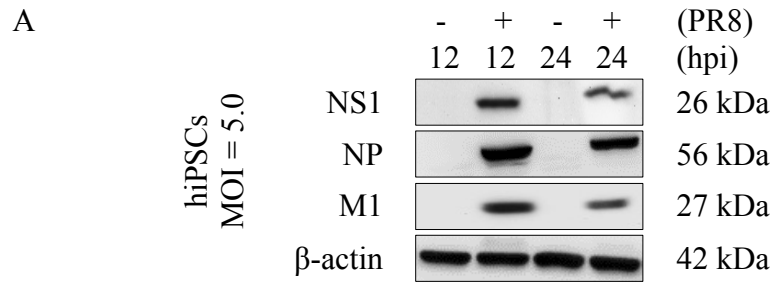


Figure 7. Production of IAV proteins at the higher MOI in human induced-pluripotent stem cells. **A)** Immunoblot detection of IAV protein production in pluripotent cells. hiPS cells were infected with influenza A/PR/8/34 at MOI = 5 PFU/cell, and protein lysates were examined for production of viral NS1, NP, and M1 proteins at 12 and 24 h post infection by Western blotting. **B)** Validation of PR8 protein production in iPS cells by immunocytochemistry. hiPS cells were infected for 24 hrs at MOI = 5 PFU/cell. After fixation, infected cells were probed with primary antibodies for viral proteins and imaged with fluorescence microscope to detect nucleoprotein (NP), non-structural protein-1 (NS1), and matrix protein 1 (M1) at 20x magnification. NP = yellow; NS1 = red; M1 = orange; nuclei = blue. Scale Bar = 50 μ m.

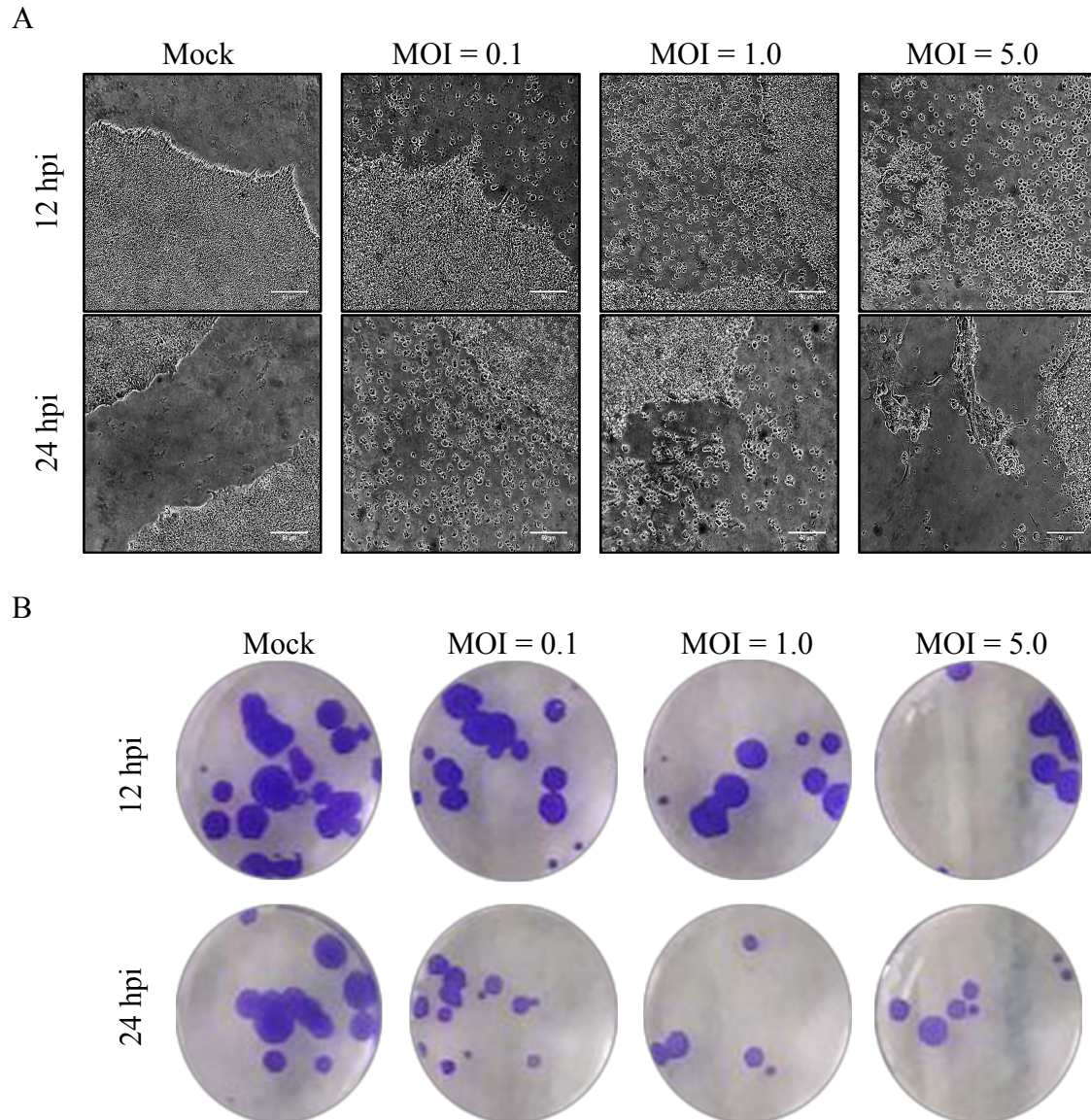
was adequate to establish an active infection in nearly 80% of cells by 24 hpi (Figure 7B). Although the assembly and maturation of infectious progeny viruses appear to be limited, these data collectively suggest that influenza A/PR/8/34 enters the nucleus of hiPSC at higher MOIs and can initiate its transcription and replication.

3.2. Influenza causes cytopathology and activates autophagy in hiPSCs

To assess potential cytolytic capacities of the virus, IAV-infected hiPSCs were assessed visually for the presence of cytopathic effect (CPE) at 12 and 24 hpi. By increasing the MOI, infected cells displayed more shrinkage, rounding up and detaching from the culture vessels, suggesting the induction of cell death. At the MOI of 5, the majority of infected cells were dissociated from their colony structures one day after infection (Figure 8A). Crystal violet staining showed a notable reduction in the number and size of colonies at 24 hpi for all three MOIs. Mock colonies of hiPSCs showed deeper staining than infected colonies due to higher cell density per colony (Figure 8B).

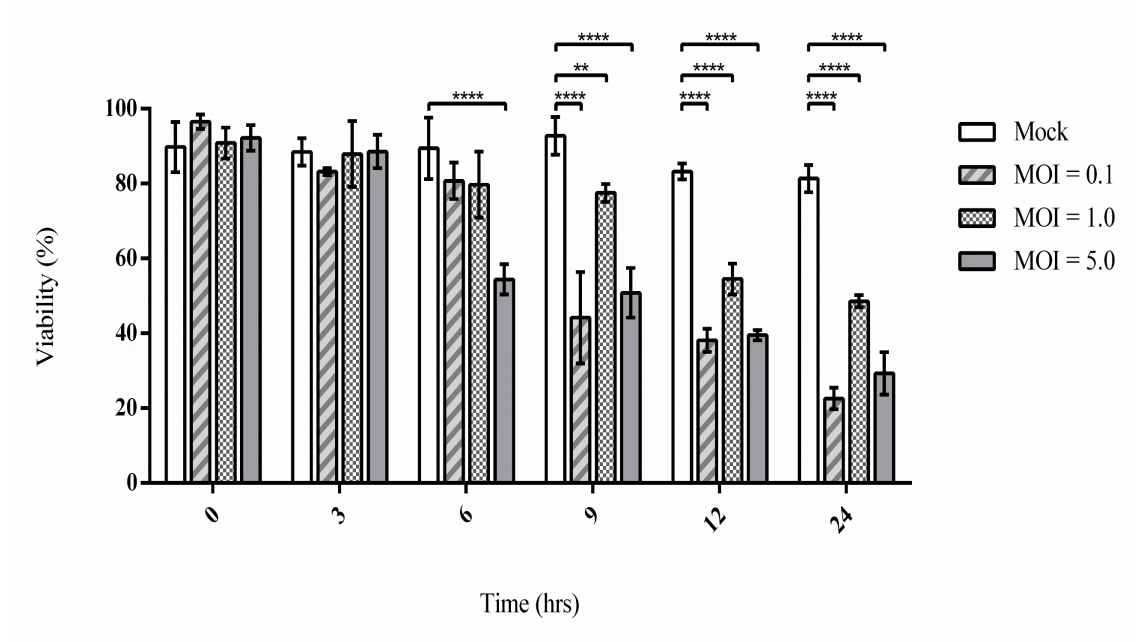
CPE development may not necessarily correlate with cell viability; therefore, trypan blue exclusion assay was performed to confirm and measure the viability of these cells at various times post-infection. In contrast to mock-infected cells, which remained more than 80% viable for a day, the viability of PR8-infected cells reduced significantly at 24 hpi for all MOIs compared to time-matched mock control as well as mock at 0 hpi (Figure 9). These results indicate the cytopathogenic entry of influenza, evident from disintegrating infected colonies and induction of cell death.

Influenza induces autophagy and apoptosis [161, 162]. To further investigate IAV-induced cell death in hiPSCs, I measured the differential expression of caspases as the main



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Figure 8. Influenza causes cytopathology in hiPSCs. A) Visual examination of IAV-induced CPE in pluripotent stem cells. Mock and virus infected cells at different MOIs were observed by the inverted microscope and photographed at 12 and 24 hpi. **B)** Impact of IAV on the size and numbers of hiPSC colonies. hiPSC colonies were infected with PR8 at three MOIs for 12 and 24 hrs, then fixed with 4% paraformaldehyde, and ultimately stained with crystal violet. Scale Bar = 50 μ m.



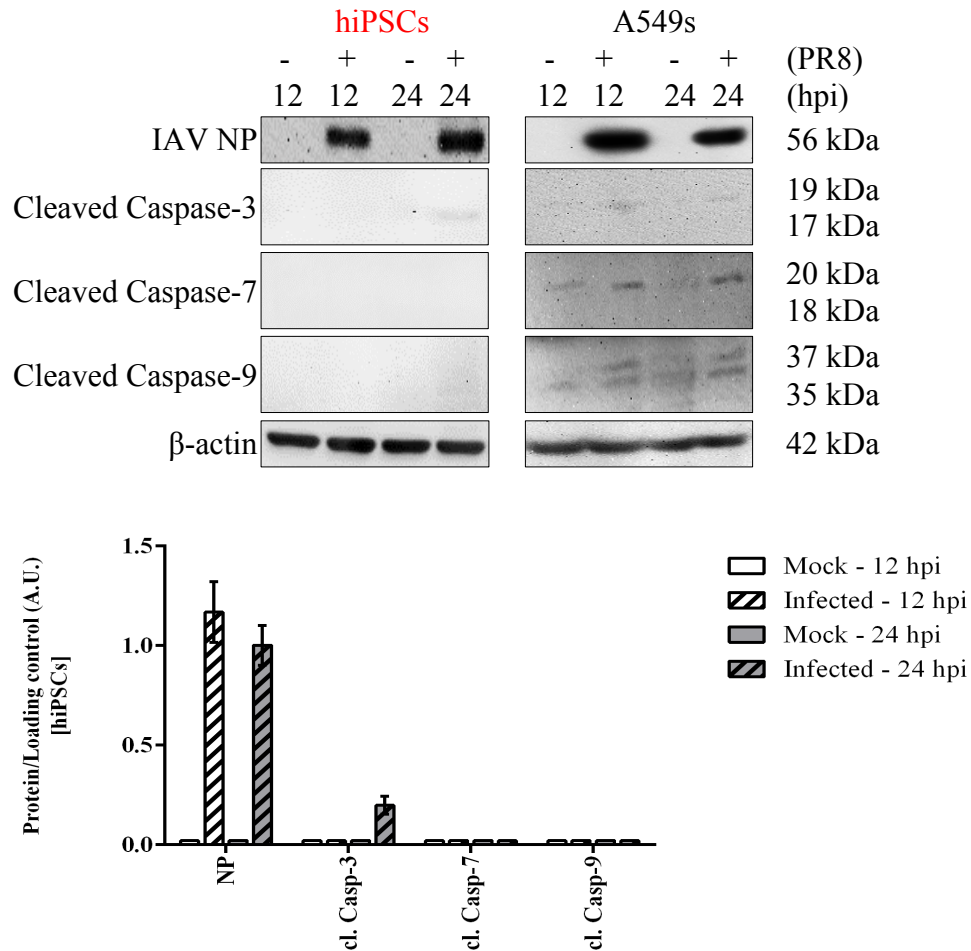
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Figure 9. PR8 infection decreases the viability of hiPSCs. The viability data was quantified based on Trypan blue exclusion test of cell viability at different MOIs and post-infection time points (P-value ≤ 0.01 = **, P-value ≤ 0.0001 = ****).

executioners of the intrinsic apoptotic pathway between mock- and PR8-infected cells by immunoblotting. IAV proteins NP, M1, and NS1 were used as markers of infection. Compared to A549 cells, PR8 infection did not elevate the cleavage of caspase-7, -3, -9 in hiPSCs by 24 hpi (Figure 10), highlighting constrained intrinsic apoptosis in pluripotent stem cells after IAV infection and the possible involvement of an alternative pathway that may promote IAV-induced cell death.

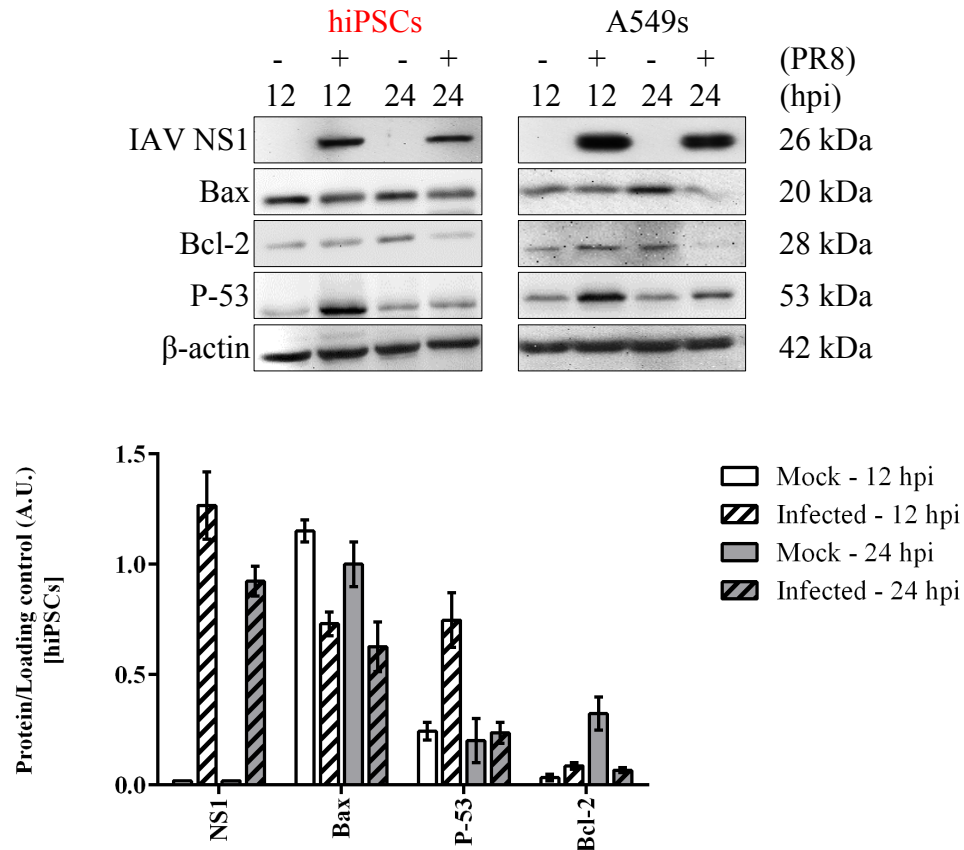
The expression of other apoptotic regulators, such as p53, Bax (pro-apoptotic), and Bcl-2 (anti-apoptotic) was examined next to better determine the unique cell-specific differences of IAV-induced apoptotic signaling in hiPSCs. In contrast to PR8-infected alveolar epithelial A549 cells which represented remarkable decrease in the expression of Bax at 24 hpi, pluripotent stem cells expressed relatively unchanged levels of this protein within 24 hrs of infection with influenza, despite the slight reduction in infected cells. The abundance of Bcl-2 was also severely decreased one day after infection in both hiPS and A549 cells. In addition, infecting these pluripotent cells with PR8 virus resulted in transient p53 accumulation only at 12 hpi without subsequent production at the later time points (Figure 11).

The increased ratio of pro-apoptotic to anti-apoptotic proteins does not correlate with IAV-induced activation of the intrinsic apoptotic pathway due to inactivation of caspases. However, similar to A549s, the induction of autophagy after PR8 infection was demonstrated by the immunoblot detection of autophagy markers LC3 β -II, Atg5, and p62 (Figure 12). Taken together, unlike limited intrinsic apoptosis, autophagy seemed to become remarkably activated in hiPSCs by IAV, which may eventually contribute in regulating an alternative cell death pathway in these cells.



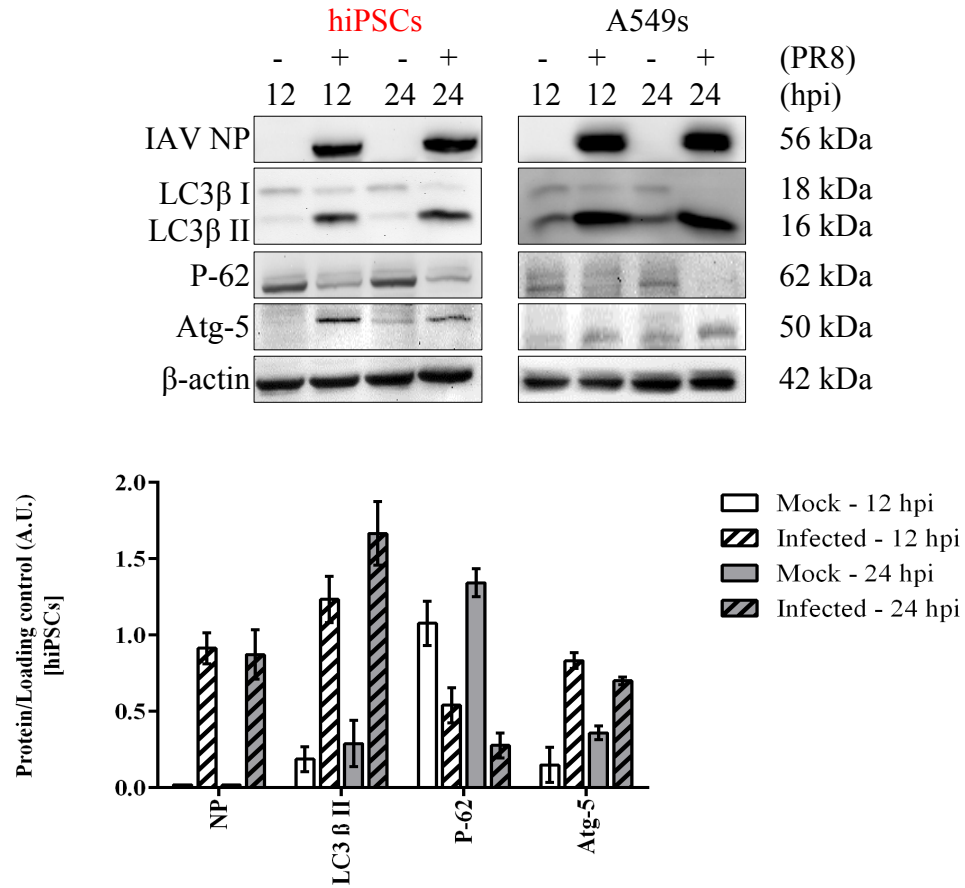
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Figure 10. IAV-triggered cell death in pluripotent stem cells is not significantly mediated by a caspase-dependent intrinsic apoptosis pathway. Following infection with PR8 virus at MOI of 5, cell lysates of pluripotent stem cells were extracted at 12 and 24 hpi, then fractionated by SDS-PAGE and assessed by Western blotting for investigating the cleavage of Caspase 3, 7, and 9. IAV protein NP was used as the marker for infection. Caspases, as the main executioners of the intrinsic apoptotic, were not found to be considerably cleaved in hiPSCs after influenza infection.



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Figure 11. Impact of IAV infection on the expression of apoptosis regulators in hiPSCs. After infecting hiPSCs with IAV at MOI of 5, proteins were extracted and assayed by immunoblotting using antibodies for measuring the expression of P-53, Bcl-2, and Bax. The production of IAV protein NS1 was considered as a marker for infection. Despite the inactivity of caspases, the increased ratio of pro-apoptotic (Bax) to anti-apoptotic (Bcl-2) proteins may suggest the contribution of caspase-independent apoptosis pathway or another type of cell death in pluripotent stem cells after influenza infection.



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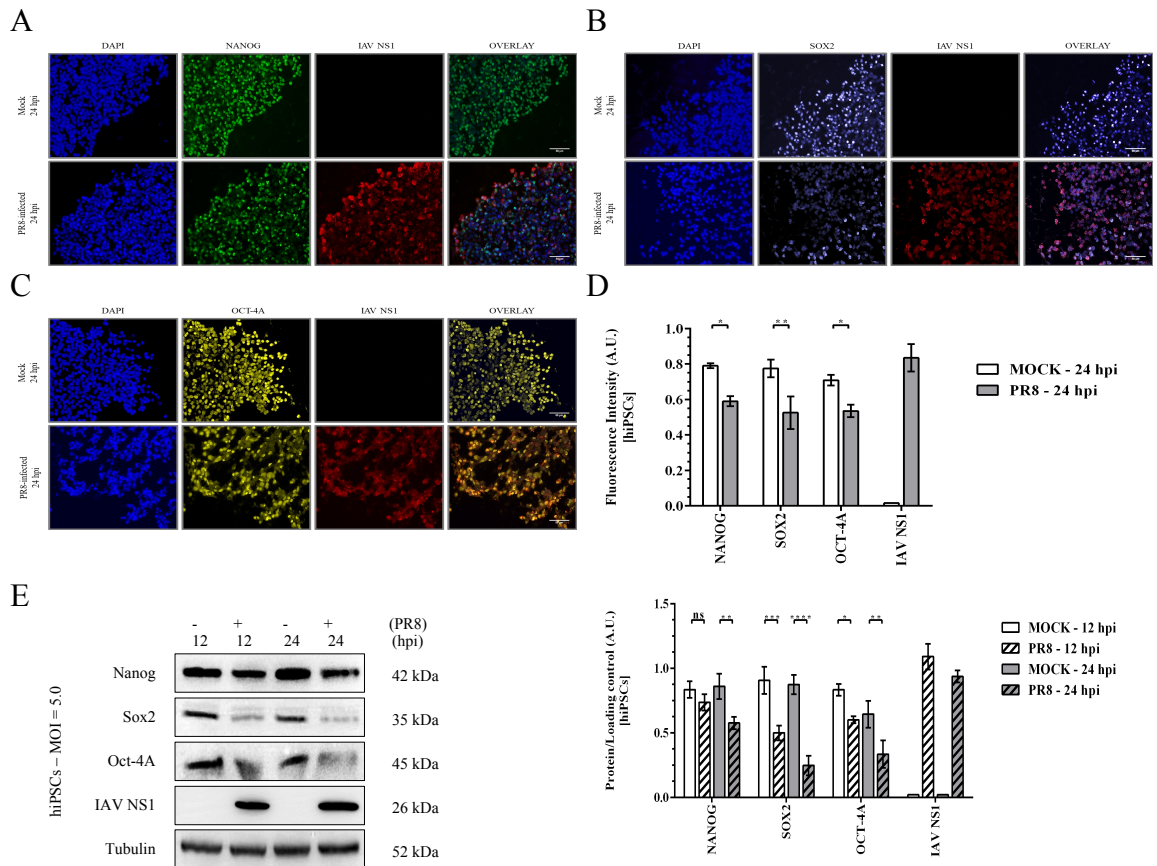
Figure 12. Influenza A virus activates autophagy in hiPSCs. Cell lysates isolated from IAV-infected hiPSCs were examined by Western blot for detecting the expression levels of markers for autophagy activation, autophagosome formation, and autophagy receptor degradation. The expression of IAV protein NP was confirmed as the marker for infection. Activation of autophagy was noted by increased accumulation of LC3β-II and Atg5 together with considerable degradation of the autophagy receptor p62.

3.3. IAV infection diminishes pluripotency of hiPS cells

The pluripotency level can be considered an essential attribute of pluripotent stem cells and represents the stemness and self-renewal ability of such cells. Utilizing immunofluorescence microscopy, I determined the expression of pluripotency markers Nanog, Sox2, and Oct-4A after PR8 infection and compared to mock-infected control cells. The intracellular detection of NS1 protein was used as a marker for productive influenza infection. Although the majority of infected cells showed pluripotency characteristics by 12 hpi (data not shown), an overall decrease of at least 20% was observed in the expression of all three pluripotency indicators after 24 hrs in influenza-infected hiPSCs at MOI of 5.0 (Figure 13D). I observed similar or greater reduction in levels of pluripotency regulators by Western blotting at 24 hpi (Figure 13E). Fluorescence signal intensity for NS1 was found to be almost equal between cells producing less Sox2 (Figure 13B) or Oct-4A (Figure 13C) and cells with a considerable level of these two proteins. However, apart from edges of infected colonies, NS1 appeared to be more abundant in cells expressing diminished levels of Nanog (Figure 13A), showing that PR8 strain is capable of decreasing the pluripotency to some extent for expanding its infectivity in differentiating hiPSCs.

3.4. Inhibition of autophagy maintains pluripotency and limits viral growth

After observing noticeable declines in the expression of proteins that control pluripotency and high levels of autophagic activity in influenza-infected hiPSCs, I attempted to establish a relationship between autophagy induction and loss of pluripotency by using autophagy regulating drugs, including Bafilomycin A1, which inhibits autophagy through both hindering vacuolar acidification required for autophagosome maturation and

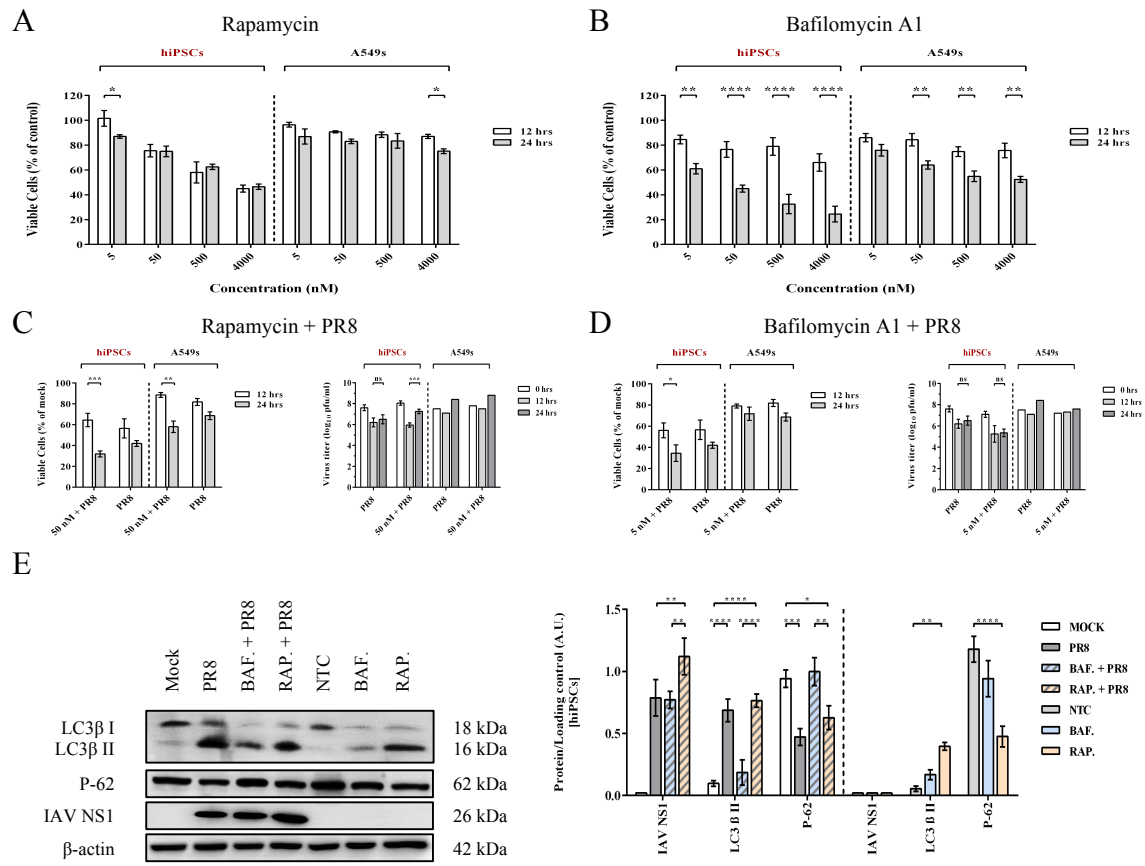


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Figure 13. IAV is capable of reducing pluripotency. The expression levels of pluripotency markers Nanog (green) (A), Sox2 (indigo) (B), and Oct-4A (yellow) (C) were measured by immunofluorescence microscopy at 24 hpi in the presence of viral NS1 protein as a marker for productive IAV infection. DNA counterstain is shown in blue (Scale Bar = 50 μ m). D) Quantification of fluorescence intensity for pluripotency regulating proteins as well as IAV NS1. Signals of detected proteins were normalized to level of nuclear staining. E) Western blot analysis validates the influenza-mediated loss of pluripotency in hiPSCs. P-value > 0.05 = ns, P-value $\leq$ 0.05 = *, P-value $\leq$ 0.01 = **, P-value $\leq$ 0.001 = ***, P-value $\leq$ 0.0001 = ****.

mTOR activation [163, 164], and Rapamycin, as an mTOR (mammalian/mechanistic target of rapamycin) inhibitor and autophagy promoter [165]. In addition to microscopic assessment of CPE (data not shown), my cytotoxicity analyses showed that more than 75% of hiPSCs can remain viable after exposure to 50 nM Rapamycin for 24 hrs (Figure 14A), while Bafilomycin A1 seemed to be more toxic for these cells, as treatment of hiPSCs with the same concentration resulted in a 50% drop in cell viability (Figure 14B). Infection with PR8 virus at MOI of 5.0 followed by treatment with 50 nM Rapamycin and 5 nM Bafilomycin A1 decreased the viability of pluripotent stem cells to below 40% within 24 hrs, which is slightly less than the effect of infection alone in the absence of these drugs (Figures 14C and 14D, left panels). To determine the effects of autophagy inducer and inhibitor on viral growth in hiPSCs, supernatants were collected and tested by plaque assay. Compared to untreated cells infected with PR8 virus, although the viral titer at 24 hrs after treatment with autophagy inducer did not pass its initial titer at time-point zero, the level of infectious progeny viruses was increased ~ 10-fold by Rapamycin (Figure 14C, right panel) but, conversely, was decreased ~ 10-fold in Bafilomycin A1-treated cells (Figure 14D, right panel), suggesting that selected concentrations of autophagy-regulating drugs can affect the viral replication by modulating influenza-induced autophagy in hiPSCs.

To confirm that the used concentrations of these drugs are effective enough to inhibit or promote autophagy, treated and non-treated cells either with or without exposure to PR8 infection, were lysed and assayed by immunoblotting to measure the expression levels of autophagy markers. Like infected non-treated cells, the conversion of LC3 β -I to LC3 β -II, as well as the degradation of P-62, were found to be significantly up-regulated in Rapamycin-treated cells in both infected and non-infected conditions, compared to mock



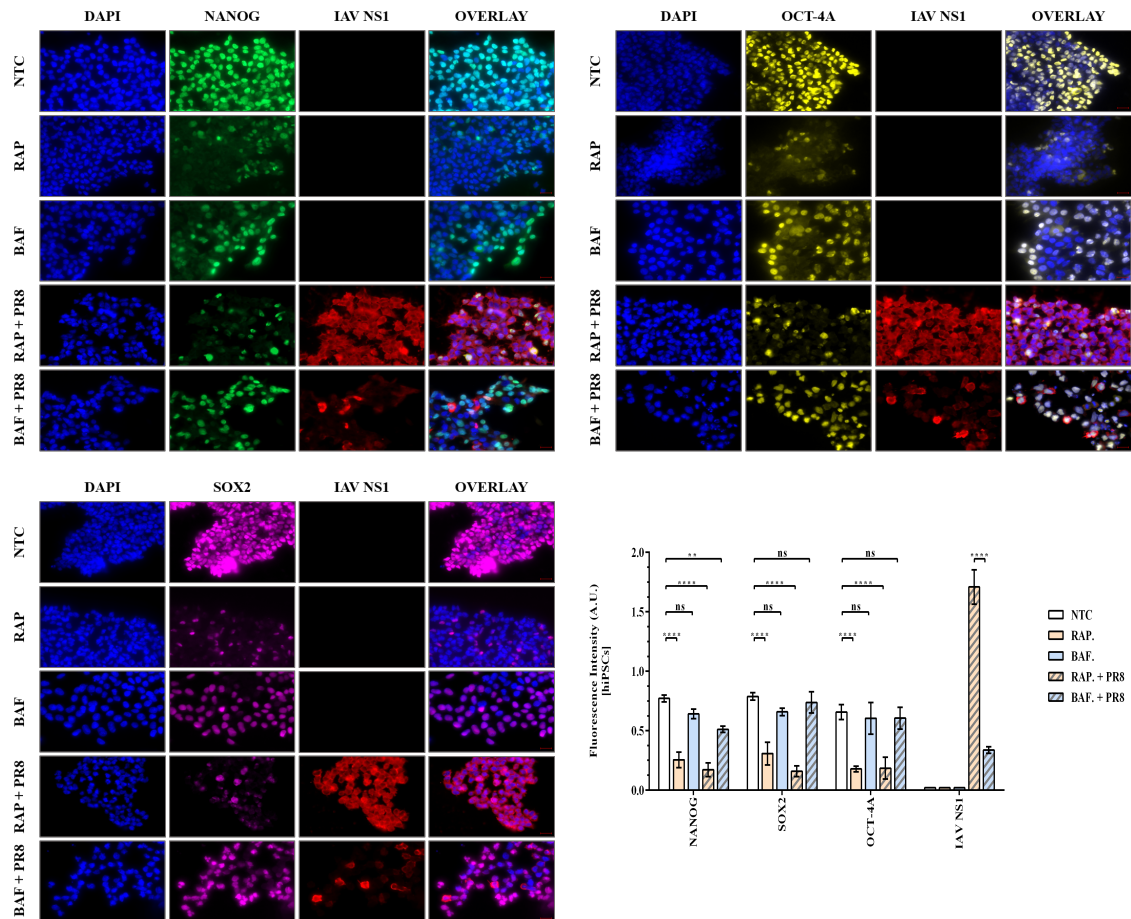
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Figure 14. Effects of autophagy inhibition and induction on IAV growth. Cytotoxicity of Rapamycin (A) and Bafilomycin A1 (B) in hiPSCs and A549s. After treating cells with various concentrations of these drugs, cell viability was assessed by WST-1 cell proliferation assay at 12 and 24 hrs. C) Rapamycin increases the influenza titer (right panel), despite reducing cell viability in presence of PR8 virus (left panel). D) Significant decline of viral titer in infected Bafilomycin A1-treated cells (right panel) is also associated with reduced cell viability (left panel). Viral titer was measured by plaque assay. E) Expression levels of autophagy markers after exposure to autophagy inducer and inhibitor in infected and non-infected hiPSCs. After treating with drugs, cell lysates were assayed

by immunoblotting for detection of LC3 β -I to II conversion and p62 degradation. P-value > 0.05 = ns, P-value $\leq$ 0.05 = *, P-value $\leq$ 0.01 = **, P-value $\leq$ 0.001 = ***, P-value $\leq$ 0.0001 = ****.

or non-treated controls, whereas the expression levels and patterns of autophagy indicators in Bafilomycin A1-treated cells, either infected or non-infected, were almost similar to the trends for mock and non-treated control (Figure 14E), which confirms the efficacy of selected concentrations in increasing or suppressing viral-induced autophagy in hiPSCs.

I then treated influenza-infected cells with these doses of Rapamycin and Bafilomycin A1 to test their effects on the level of pluripotency. Analysis of fluorescence signal intensities for both infected and non-infected Rapamycin-treated cells confirmed 60% or more loss in the expression of proteins controlling pluripotency network, including Nanog, Sox2, and Oct-4, which is more substantial than the effect of normal infection without drug treatment (Figures 15 and 13). In Bafilomycin A1-treated cells, the levels of pluripotency proteins did not change significantly, but infection with PR8 virus caused slight reduction in their expressions, which were still between 2- to 4-fold greater than signals for Rapamycin-treated cells, showing that Bafilomycin A1 reduces the negative effect of influenza-mediated autophagy on pluripotency. Moreover, although my Western blotting indicated that Bafilomycin A1-treated cells can express IAV NS1 protein almost equal to infected non-treated cells, I found that fluorescence signals for NS1 production were severely decreased under the influence of Bafilomycin A1, while the NS1 expression was highly increased by Rapamycin based on both assays, compared to normal non-treated infected cells (Figures 15, 14E, and 13). Since both pharmacological promotion of autophagy by Rapamycin and influenza upregulate autophagic activity by inhibiting mTOR, these results suggest a relationship between influenza-induced autophagy and loss of pluripotency.



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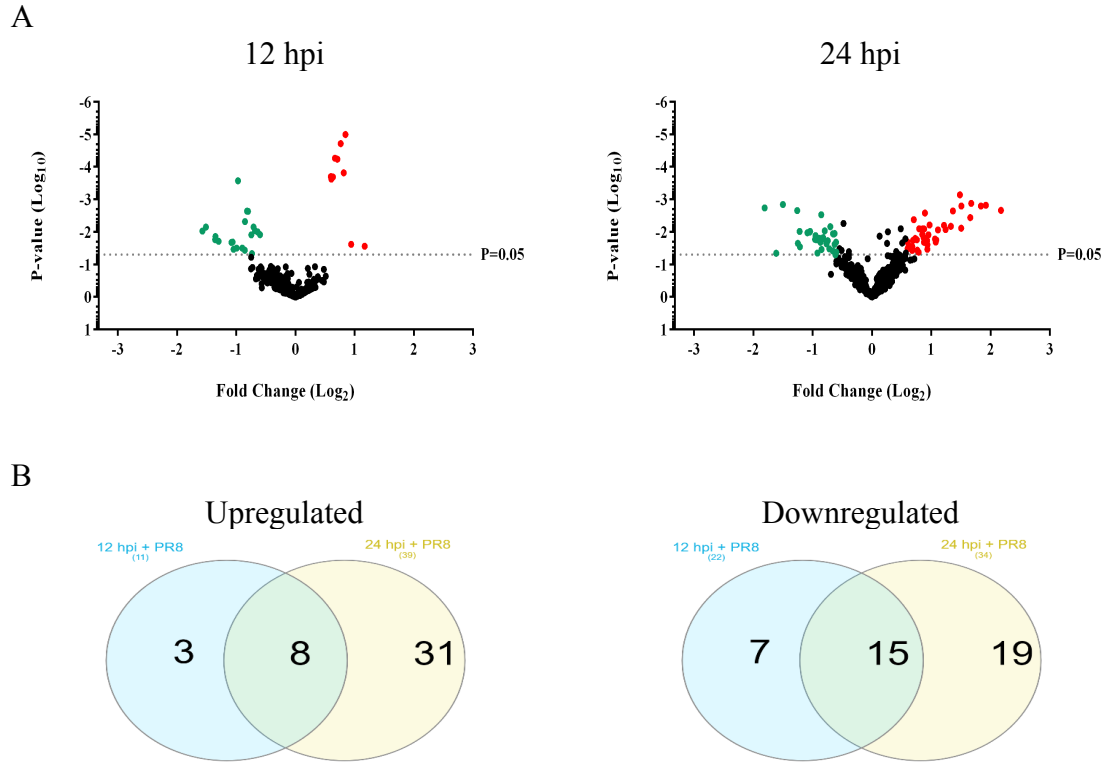
Figure 15. Effects of autophagy inhibition and induction on pluripotency. Induction of autophagy negatively affects the pluripotency, while the inhibition of autophagic activity maintains this characteristic of pluripotent stem cells. The expression of pluripotency proteins Nanog (green), Sox2 (violet), and Oct-4A (yellow) was determined by immunofluorescence microscopy at 24 hpi in presence of viral NS1 protein as a marker for infection. DNA counterstain is shown in blue. Fluorescence signals were normalized to nuclear staining. P-value ≤ 0.05 = *, P-value ≤ 0.01 = **, P-value ≤ 0.001 = ***, P-value ≤ 0.0001 = ****. Scale Bar = 20 μ m.

3.5. PR8 infection induces dysregulations in the hiPSC proteome

I performed proteomic analysis of three separate biological replicates using SOMAScan[®], a novel multi-plexed system that allowed simultaneous analysis of all experimental conditions, to evaluate changes in the expression of intracellular proteins related to various biological processes. In total, 1,307 proteins were screened in lysates isolated from IAV-infected hiPSCs at the MOI of 5.0. To sort out modified proteins, I calculated z-scores separately for each biological replicate to normalize all screened proteins at a 95% confidence level cutoff. Z-scores values of $\geq 1.96\sigma$ and $\leq -1.96\sigma$ were specified as the criteria for up- and down-regulation patterns.

Significant alterations were observed in the expression of 33 proteins at 12 hpi, while my data demonstrated higher numbers of dysregulated proteins at 24 hpi than at 12 hpi, as 39 proteins were significantly upregulated and 34 were significantly downregulated (Figure 16A, Table 2). Venn diagrams specifying the number of overlapping proteins indicated that 8 common upregulated and 15 common downregulated proteins followed their altered pattern of expression between datasets of differentially expressed proteins at 12 and 24 hpi (Figure 16B). The protein abundance heat map and clustering analysis showed that about 6% of the measured proteome underwent significant modifications after influenza infection (Figure 17).

To test the validity of my proteomic screening, I confirmed the differential regulations of some proteins by Western blotting. These proteins were selected because of their large fold-changes and availability of antibodies. These proteins include serotransferrin (TF), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and proteasome subunit alpha



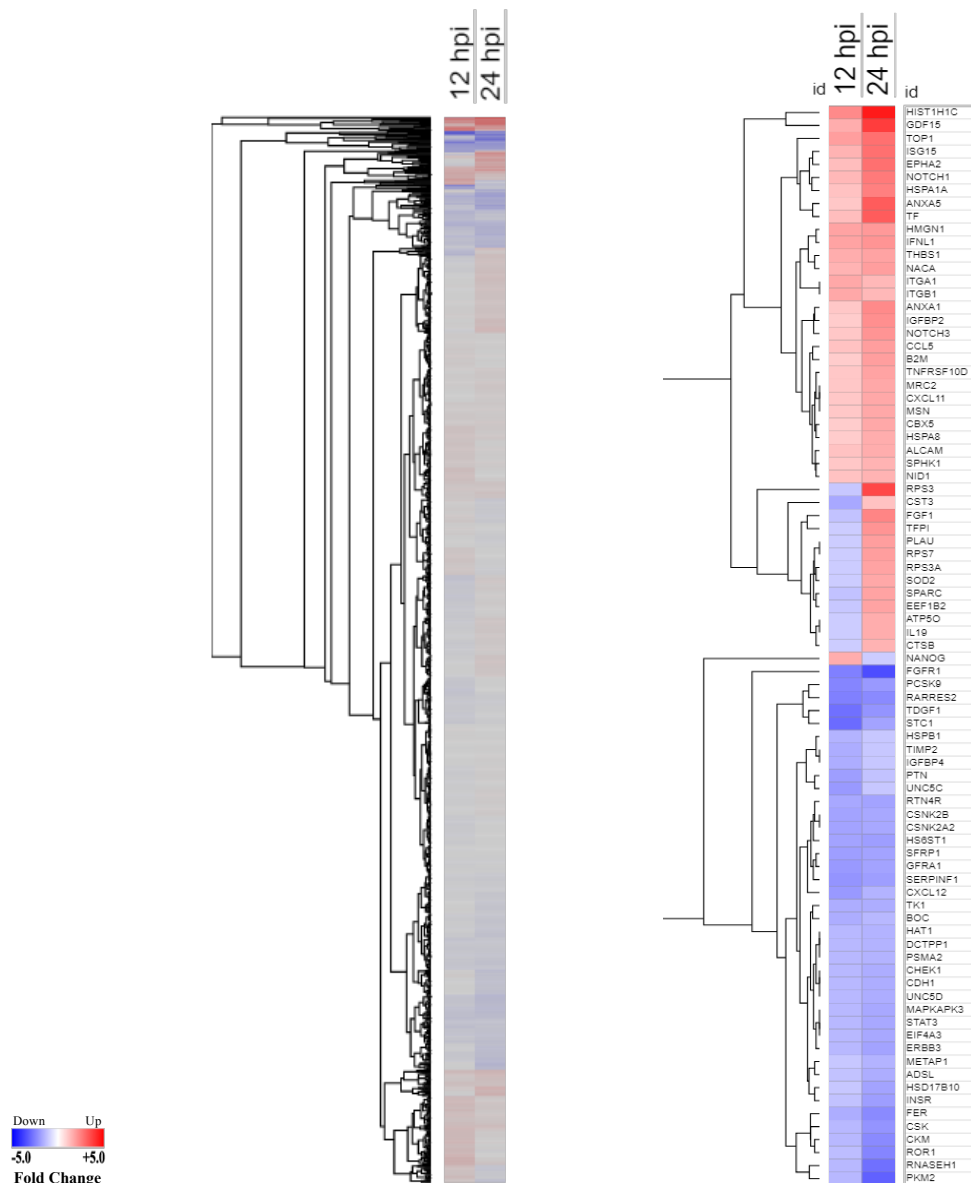
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Figure 16. Overall statistics from SOMAScan proteomic analysis of IAV-infected hiPSCs at MOI of 5.0. **A)** Volcano plots showing the expression fold changes and the significance of differentially expressed proteins at 12 and 24 hpi ($P\text{-value} < 0.05$). More dysregulated proteins were observed at 24 hpi. **B)** Venn diagram of the overlapping significantly regulated proteins between two time-points. Diagrams of significantly expressed proteins were plotted using InteractiVenn free online software. About 16% of upregulated and 27% of downregulated proteins continued their altered pattern of expression to the second time-point.

Table 2. hiPS cell proteins affected by PR8 infection.

Entrez Gene Symbol	Protein Name	12 hpi		24 hpi	
		Fold Change	P-value	Fold Change	P-value
HIST1H1C	histone cluster 1 H1 family member c	2.25	2.72E-02	4.52	2.16E-03
TOP1	DNA topoisomerase I	1.92	2.37E-02	2.83	7.57E-03
HMGN1	high mobility group nucleosome binding domain 1	1.80	1.00E-05	1.96	6.10E-03
IFNL1	interferon lambda 1	1.76	1.51E-04	2.08	1.64E-02
ITGA1	integrin subunit alpha 1	1.70	1.90E-05	1.47	5.45E-02
ITGB1	integrin subunit beta 1	1.70	1.90E-05	1.47	5.45E-02
GDF15	growth differentiation factor 15	1.64	5.70E-05	3.78	1.51E-03
THBS1	thrombospondin 1	1.59	5.40E-05	1.84	8.02E-03
NANOG	Nanog homeobox	1.55	2.03E-04	-1.06	6.03E-01
NACA	nascent polypeptide-associated complex alpha subunit	1.52	2.34E-04	1.90	1.83E-02
ISG15	ISG15 ubiquitin-like modifier	1.52	1.97E-04	2.84	1.58E-03
HSPB1	heat shock protein family B (small) member 1	-1.51	1.20E-02	-1.10	4.54E-01
TIMP2	TIMP metalloproteinase inhibitor 2	-1.56	9.90E-03	-1.10	3.49E-01
BOC	BOC cell adhesion associated, oncogene regulated	-1.61	9.02E-03	-1.42	1.18E-01
IGFBP4	insulin like growth factor binding protein 4	-1.63	6.99E-03	-1.14	2.80E-01
FER	FER tyrosine kinase	-1.66	4.61E-02	-2.38	2.23E-02
TK1	thymidine kinase 1	-1.67	1.22E-02	-1.62	1.37E-02
RNASEH1	ribonuclease H1	-1.68	1.00E-05	-2.83	1.42E-03
CST3	cystatin C	-1.74	2.29E-03	1.20	5.34E-02
RTN4R	reticulon 4 receptor	-1.76	2.26E-03	-1.84	1.56E-02
CSNK2B	casein kinase 2 beta	-1.80	4.76E-03	-1.78	1.61E-02
CSNK2A2	casein kinase 2 alpha 2	-1.80	4.76E-03	-1.78	1.61E-02
HS6ST1	heparan sulfate 6-O-sulfotransferase 1	-1.81	3.62E-02	-1.91	1.73E-02
PTN	pleiotrophin	-1.86	3.13E-02	-1.20	1.57E-01
SFRP1	secreted frizzled related protein 1	-1.96	2.67E-04	-1.80	5.91E-03
UNC5C	unc-5 netrin receptor C	-1.99	3.14E-02	-1.15	2.83E-01
GFRA1	GNDF family receptor alpha 1	-2.06	3.40E-02	-1.80	3.44E-02
CXCL12	C-X-C motif chemokine ligand 12	-2.08	2.03E-02	-1.54	2.21E-02
SERPINF1	serpin family F member 1	-2.11	2.08E-02	-1.94	1.68E-02
PCSK9	proprotein convertase subtilisin/kexin type 9	-2.46	1.93E-02	-2.07	9.92E-03
RARRES2	retinoic acid receptor responder 2	-2.55	1.37E-02	-2.33	9.53E-03
FGFR1	fibroblast growth factor receptor 1	-2.56	1.70E-02	-3.50	1.81E-03
TDGF1	teratocarcinoma-derived growth factor 1	-2.85	6.99E-03	-2.12	1.05E-02
STC1	stanniocalcin 1	-2.97	9.27E-03	-1.89	4.41E-02
RPS3	ribosomal protein S3	-1.13	5.69E-01	3.58	1.58E-03
ANXA5	annexin A5	1.14	3.55E-01	3.19	1.31E-03
TF	transferrin	1.39	3.40E-01	3.16	3.58E-03
EPHA2	EPH receptor A2	1.39	1.40E-01	2.80	7.14E-04
NOTCH1	notch 1	1.42	2.29E-01	2.58	2.26E-03
HSPA1A	heat shock protein family A (Hsp70) member 1A	1.25	1.17E-01	2.52	6.64E-03
FGF1	fibroblast growth factor 1	-1.28	3.95E-01	2.36	8.33E-03
ANXA1	annexin A1	1.10	6.39E-01	2.32	6.23E-03
IGFBP2	insulin like growth factor binding protein 2	1.00	9.94E-01	2.17	8.64E-03
TFPI	tissue factor pathway inhibitor	-1.05	8.21E-01	2.12	1.76E-02
NOTCH3	notch 3	1.19	4.36E-01	2.11	2.02E-02
CCL5	C-C motif chemokine ligand 5	1.24	2.87E-01	1.94	1.23E-02
PLAU	plasminogen activator, urokinase	-1.00	9.80E-01	1.91	2.46E-02
RPS7	ribosomal protein S7	-1.00	9.77E-01	1.91	3.39E-02
B2M	beta-2-microglobulin	1.04	8.52E-01	1.86	2.61E-03
TNFRSF10D	TNF receptor superfamily member 10d	1.17	1.99E-01	1.84	2.06E-02
SPARC	secreted protein acidic and cysteine rich	-1.23	2.02E-01	1.83	1.48E-02
RPS3A	ribosomal protein S3A	-1.03	8.53E-01	1.81	8.07E-03
EEF1B2	eukaryotic translation elongation factor 1 beta 2	-1.10	6.86E-01	1.80	1.19E-02
MRC2	mannose receptor C type 2	1.15	5.09E-01	1.74	7.98E-03
CBX5	chromobox 5	1.08	6.93E-01	1.73	4.16E-02
CXCL11	C-X-C motif chemokine ligand 11	1.11	6.10E-01	1.70	3.60E-02
SOD2	superoxide dismutase 2	-1.08	5.51E-01	1.69	1.74E-02
MSN	moesin	1.14	5.16E-01	1.66	1.57E-02
ATP5O	ATP synthase peripheral stalk subunit OSCP	-1.08	7.09E-01	1.63	8.31E-03
IL19	interleukin 19	-1.05	7.26E-01	1.62	1.81E-02
ALCAM	activated leukocyte cell adhesion molecule	1.29	2.87E-01	1.60	3.65E-02
HSPA8	heat shock protein family A (Hsp70) member 8	1.07	5.76E-01	1.60	2.78E-02
SPHK1	sphingosine kinase 1	1.17	5.26E-01	1.54	2.56E-02
NID1	nidogen 1	1.25	3.90E-01	1.52	3.21E-02
CTSB	cathepsin B	-1.01	9.41E-01	1.52	2.02E-02
HAT1	histone acetyltransferase 1	-1.45	2.25E-01	-1.52	4.12E-02
DCTPP1	dCTP pyrophosphatase 1	-1.44	2.73E-01	-1.52	4.11E-02
METAP1	methionyl aminopeptidase 1	-1.12	6.67E-01	-1.53	4.97E-02
PSMA2	proteasome subunit alpha 2	-1.43	2.07E-01	-1.55	2.39E-02
CHEK1	checkpoint kinase 1	-1.48	1.32E-01	-1.57	2.32E-02
CDH1	cadherin 1	-1.39	2.16E-01	-1.58	2.24E-02
ADSL	adenylosuccinate lyase	-1.19	2.60E-01	-1.58	3.77E-02
UNC5D	unc-5 netrin receptor D	-1.45	1.23E-01	-1.64	3.28E-02
MAPKAPK3	mitogen-activated protein kinase-activated protein kinase 3	-1.41	2.15E-01	-1.68	1.84E-02
STAT3	signal transducer and activator of transcription 3	-1.40	1.73E-01	-1.70	2.30E-02
EIF4A3	eukaryotic translation initiation factor 4A3	-1.39	2.23E-01	-1.74	9.16E-03
ERBB3	erb-b2 receptor tyrosine kinase 3	-1.43	1.39E-01	-1.77	1.49E-02
HSD17B10	hydroxysteroid 17-beta dehydrogenase 10	-1.11	6.21E-01	-1.82	2.06E-02
INSR	insulin receptor	-1.38	2.73E-01	-1.93	1.28E-02
CSK	C-terminal Src kinase	-1.58	2.76E-01	-2.06	9.52E-03
CKM	creatine kinase, M-type	-1.48	3.88E-01	-2.32	2.85E-02
ROR1	receptor tyrosine kinase like orphan receptor 1	-1.35	2.60E-01	-2.39	2.18E-03
PKM2	pyruvate kinase M1/2	-1.48	5.20E-01	-3.06	4.48E-02

Values shaded in gray are indicating the fold changes of significantly differentially expressed proteins based on the cutoff of +1.5 and -1.5 for up- and down-regulation, respectively. Published in Cell Death & Disease (Zahedi-Amiri et al. 2019) [160]

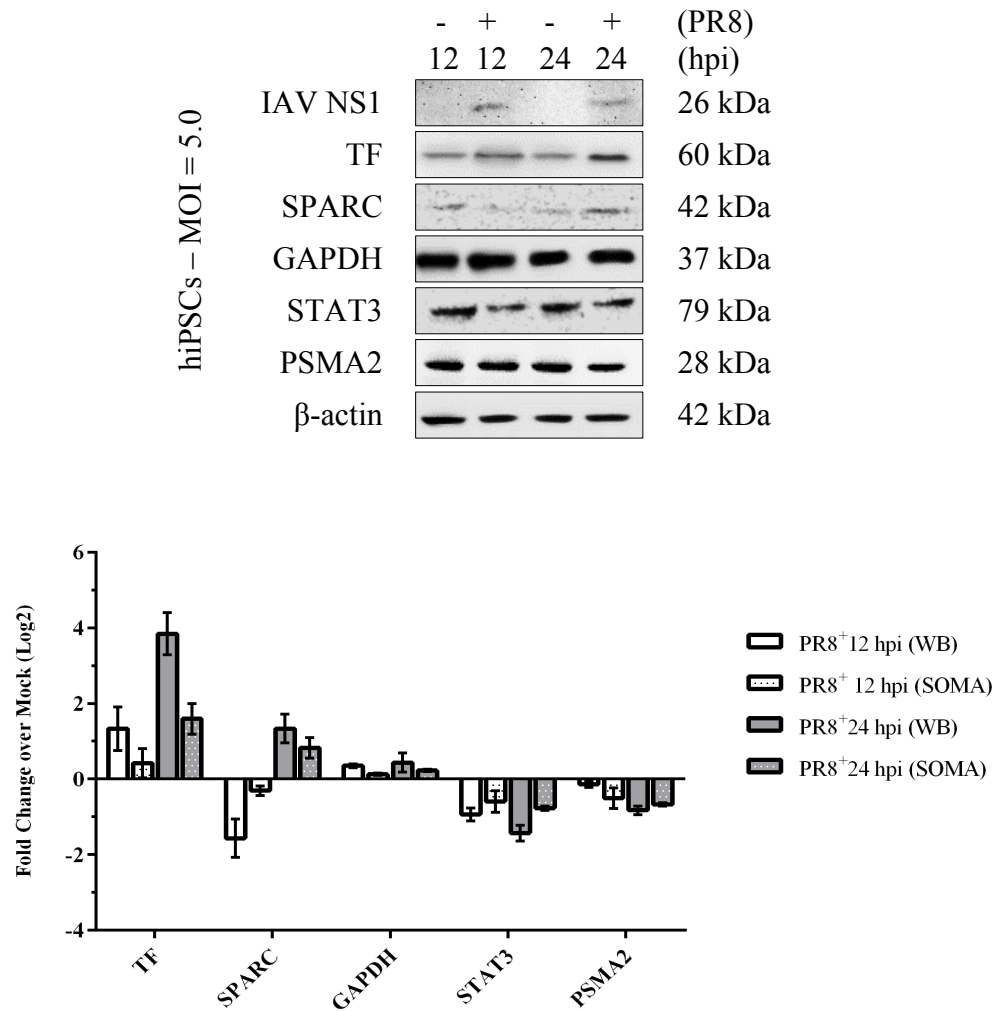


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Figure 17. The heatmap of the whole proteome (left) and the comparison (right) heatmap of differentially expressed proteins between 12 and 24 hpi. Heatmaps were generated by uploading fold changes information from the entire analyzed proteome as well as from differentially expressed proteins into MORPHEUS online software. Almost 6% of the screened proteome showed significant alterations following influenza infection.

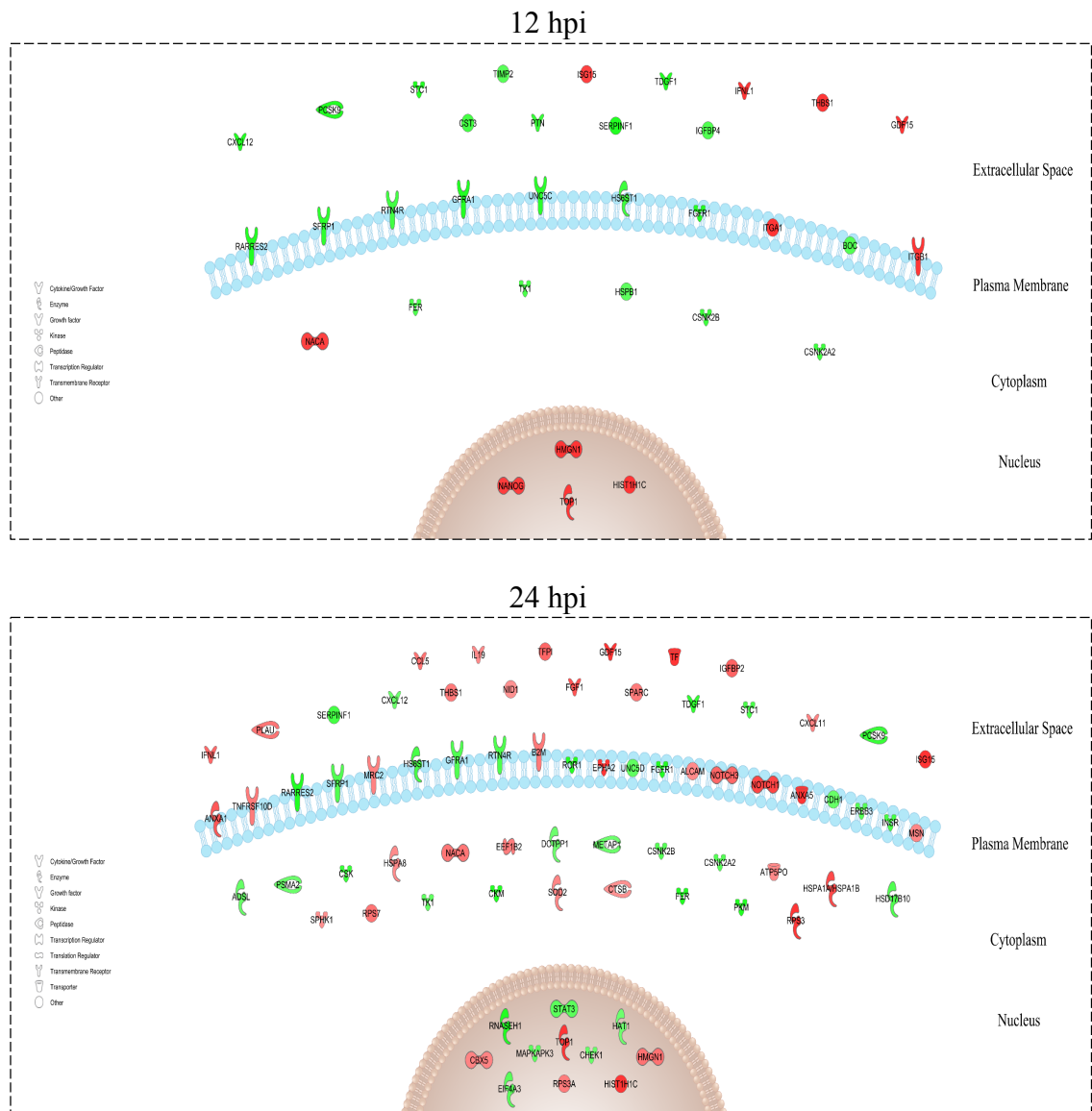
type-2 (PSMA2). Influenza NS1 also was probed to confirm successful infection. Compared to fold-changes acquired from the SOMAScan experiment, densitometry analyses of immunoblots revealed similar expression levels for all selected proteins (Figure 18).

The Ingenuity Pathways Analysis (IPA) tool was used to illustrate the subcellular localization of regulated proteins. As shown in Figure 19, most of the affected proteins at 12 hpi could be mapped in extracellular (secretory) (39.3%) and plasma membrane (30.3%) regions. Furthermore, these two areas together contained 54.7% of influenza-modulated proteins at 24 hpi, even though cytoplasmic proteins had the highest share of differentially expressed proteins from this time point.



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Figure 18. Western blot confirmation of selected differentially regulated proteins from SOMAScan results. Western blot results are represented from multiple different gels. Various loading control band intensities information were used from different blots to perform densitometry analysis. Immunoblots indicated expression patterns similar to fold-changes obtained from the SOMAScan screening for all selected proteins.



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Figure 19. The subcellular localizations of regulated proteins at 12 and 24 hpi. Graphical representation of cellular compartments was illustrated using Pathway designer plugin in the IPA tool. Up- and down-regulated proteins are highlighted in red and green, respectively.

3.6. Influenza targets proteins controlling embryogenesis and differentiation

Differentially expressed proteins in response to PR8 infection at 12 hpi and 24 hpi were classified based on gene ontology analyses using GOTERM and PANTHER database in three principal groups, including biological processes, molecular functions, and protein classes. Analysis was done by both functional classification and statistical enrichment tests, which provide information in the format of main categories and subcategories, respectively.

In functional classification analysis, despite the involvement of several main categories for biological processes like cellular process (45.5%), developmental process (18.2%), response to stimulus (27.3%), etc., more than half of up-regulated proteins at 12 hpi could be attributed to metabolic categories (Figure 20A-upper section). Similarly, 45% and 40% of up-regulated proteins at 24 hpi belonged to cellular process and metabolic activities, respectively (Figure 20B- upper section). These two GO categories also had the highest proportions of down-regulated proteins at both 12 hpi and 24 hpi (Figure 20C and 20D, upper sections). Of 11 up-regulated proteins at 12 hpi, 8 were recognized as binding molecules through GO molecular function classification (Figure 20A-middle section), whereas up-regulated proteins at 24 hpi were not only assigned to binding function, but also to other functional terms, such as catalytic activity, structural molecule activity, and receptor activity (Figure 20B-middle section). Likewise, down-regulated proteins were distributed among the same functional GO terms (most notably binding and catalytic activity) for 12 hpi (Figure 20C-middle section) and 24 hpi (Figure 20D-middle section) datasets. PANTHER analyses of protein classes for influenza-altered proteins are depicted in lower sections of Figure 20A-D. Briefly, proteins belonging to nucleic acid binding class

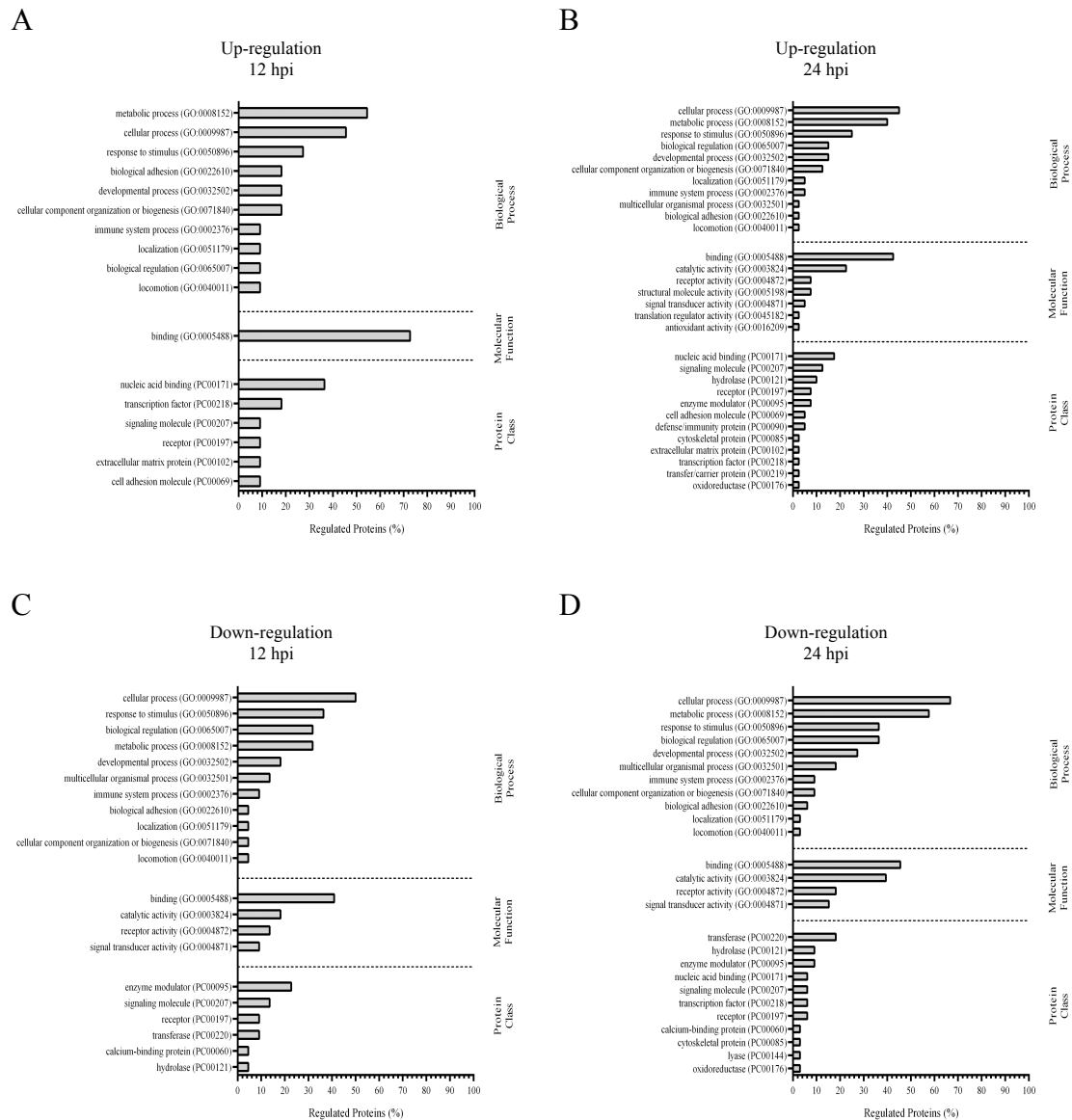


Figure 20. Functional classification analysis of up- and down-regulated proteins. Lists of up- (A & B) and down-regulated (C & D) protein IDs were inserted into GOTERM and PANTHER databases separately and examined for associations with main categories belonging to biological processes, molecular functions, and protein classes.

were more up-regulated either at 12 hpi (Figure 20A-lower section) or 24 hpi (Figure 20B-lower section), while enzyme modulator class, as a term with the highest numbers of molecules, encompassed 22.7% of down-regulated proteins at 12 hpi (Figure 20C-lower section). Given that further down-regulated proteins were seen at 24 hpi, I could identify a few more protein class terms for these molecules compared to 12 hpi; however, transferase and hydrolase ontology classes included more proteins rather than some other classes with single members (Figure 20D-lower section).

According to statistical enrichment analysis, various subcategories for biological processes were classified, but almost one third of highly enriched ontological terms from up-regulated proteins at 12 hpi could be attributed to classes that are related to differentiation and embryogenesis, such as dorsal/ventral axis specification, embryo development, pattern specification, and ectoderm development (Figure 21A-upper section). However, the vast majority of these developmental terms were not enriched significantly at 24 hpi, and up-regulated proteins at this time point showed top enrichment scores for other biological processes, like transmembrane receptor protein tyrosine kinase signaling pathway, protein folding, response to interferon gamma, cytokine-mediated signaling pathway, MAPK cascade, negative regulation of apoptosis, etc. (Figure 21B-upper section). Common GO-based biological processes among all upregulated proteins at both 12 and 24 hpi include chromatin remodeling, DNA replication, protein phosphorylation, chromatin organization, response to abiotic stimulus, and chromosome segregation. None of these common terms indicated higher enrichment scores at 24 hpi, except for the response to abiotic stimulus that appeared with slightly higher score, compared to 12 hpi.

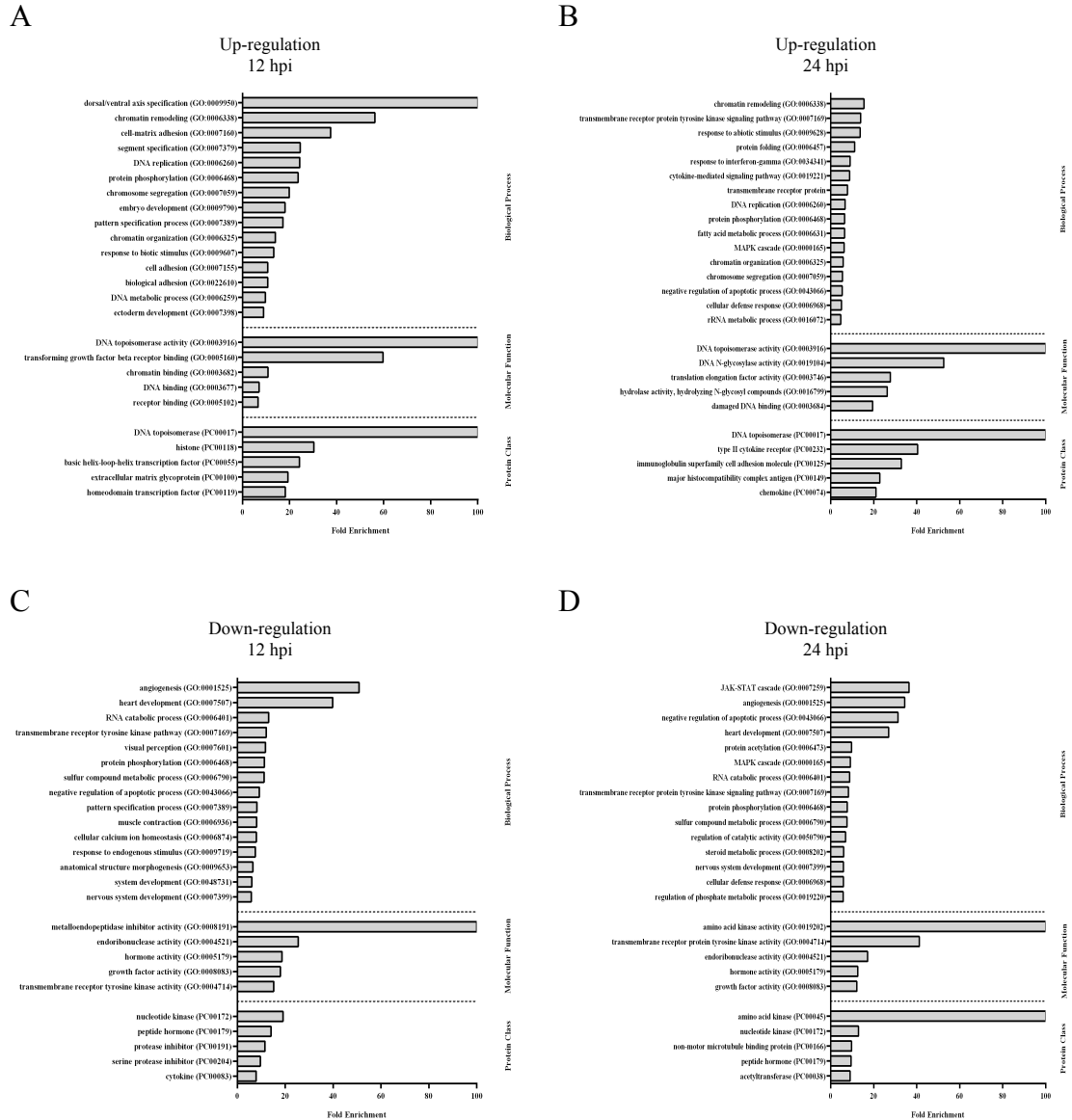


Figure 21. Statistical enrichment analysis of up- and down-regulated proteins. IDs of up- (A & B) and down-regulated (C & D) protein were uploaded into GOTERM and PANTHER databases individually and analyzed based on subcategories from different biological processes, molecular functions, and protein classes.

Similarly, downregulated proteins also corresponded to pathways regulating differentiation and embryogenesis. Based on downregulated proteins, the common biological processes between 12 and 24 hpi time points include angiogenesis, heart development, negative regulation of apoptotic process, RNA catabolic process, protein phosphorylation, transmembrane receptor tyrosine kinase signalling pathway, sulfur compound metabolic process, and nervous system development. Time-point specific top enriched biological processes at 12 hpi consisted of visual perception, pattern specification process, muscle contraction, cellular calcium ion homeostasis, response to endogenous stimulus, anatomical structure morphogenesis, and system development (Figure 21C-upper section). Biological processes that were exclusively enriched at 24 hpi were comprised of JAK-STAT cascade, protein acetylation, MAPK cascade, regulation of catalytic activity, steroid metabolic process, cellular defense response, and regulation of phosphate metabolic process (Figure 21D- upper section).

GO molecular function classification of upregulated proteins revealed that the highest enrichment score belongs to DNA topoisomerase activity at both 12 and 24 hpi. Nevertheless, other top four enriched molecular functions at these time points did not show common ontological terms (Figure 21A and 21B-middle sections). In contrast, classification of downregulated proteins for molecular functions showed four common terms among top five enriched functions at 12 and 24 hpi, which include endoribonuclease activity, hormone activity, growth factor activity, and transmembrane receptor tyrosine kinase activity (Figure 21C and 21D-middle sections). The metalloendopeptidase inhibitor activity and amino acid kinase activity were identified as time-point specific as well as top

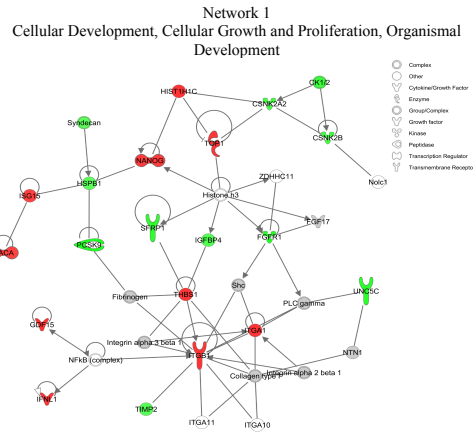
enriched molecular functions based on downregulated proteins at 12 and 24 hpi, respectively.

PANTHER analyses of protein classes for upregulated proteins determined DNA isomerase as the highest enriched protein class at both tested time points (Figure 21A and 21B-lower sections). No other common protein classes were recognized among top five enriched terms from upregulated proteins at 12 and 24 hpi. Other unique protein classes that were enriched at 12 hpi include histone, basic helix-loop-helix transcription factor, extracellular matrix glycoprotein, and homeodomain transcription factor. At 24 hpi, classification of upregulated proteins was associated with higher enrichment for other classes, such as type II cytokine receptor, immunoglobulin superfamily cell adhesion, major histocompatibility complex antigen, and chemokine. Analysis of downregulated proteins indicated that nucleotide kinase and peptide hormone are common protein classes between 12 and 24 hpi time points (Figure 21C and 21D-lower sections).

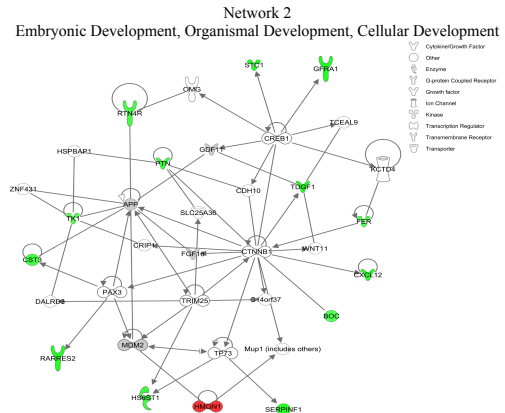
3.7. Influenza affects protein-protein interaction network of embryonic development

To gain a better understanding of interactions among dysregulated proteins obtained at each time point in influenza-infected pluripotent stem cells, interconnecting networks were generated based on direct relationships only by uploading datasets containing protein IDs, fold changes compared to mock-infected cells and p-values into the IPA program. For the 12 hpi time point, the 2 top networks that comprised 14 or more focus members were related to cellular development, cellular growth and proliferation, organismal development (p-score=49), and embryonic development pathways (p-score=33) (Figure 22A). Due to higher numbers of differentially expressed proteins at 24 hpi, network analyses showed

A

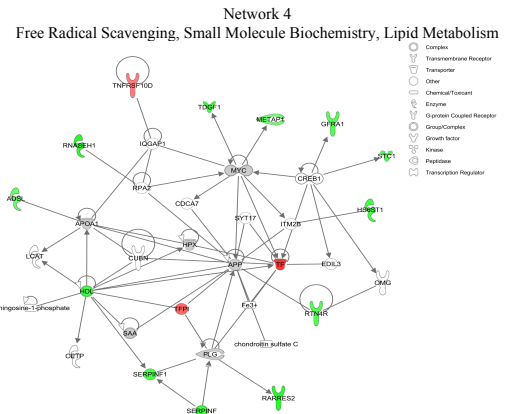
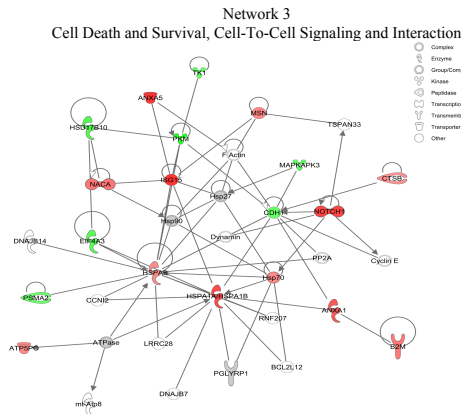
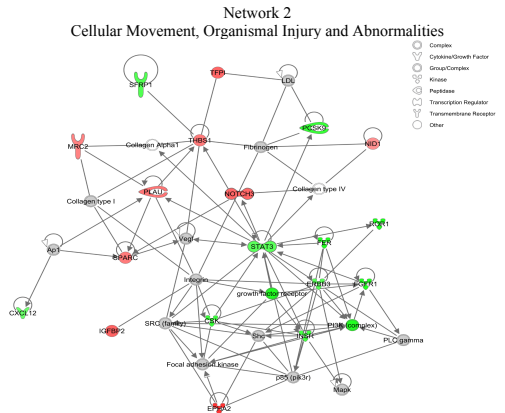
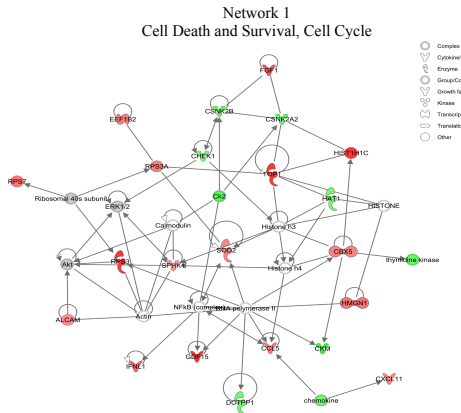


12 hpi



B

24 hpi



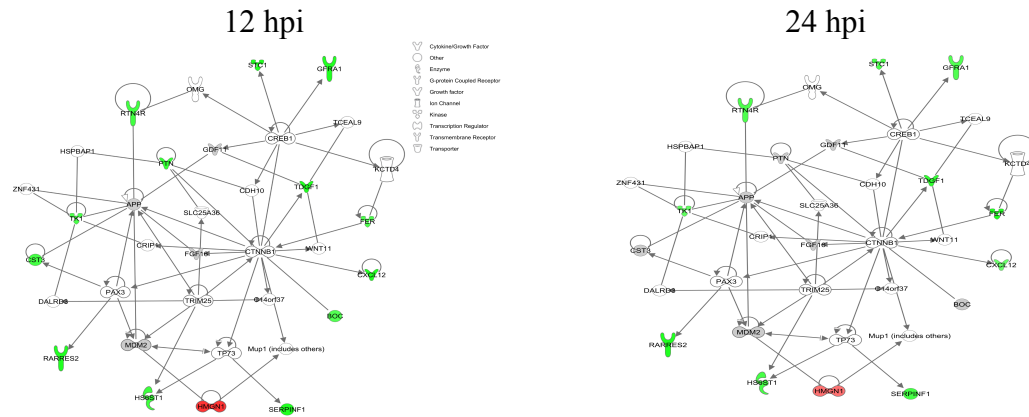
more extreme in dataset
 red Increased measurement
 green Decreased measurement

Figure 22. Molecular networks of regulated proteins. The datasets containing protein IDs, fold changes, and P-values were imported into the IPA software, and interacting networks were assembled for differentially expressed proteins at 12 hpi (A) and 24 hpi (B); Up- and down-regulated proteins are indicated in red and green, respectively; gray proteins denote that they were identified in this study but not affected; colorless proteins interact with various proteins in the pathway but were not recognized in my screening.

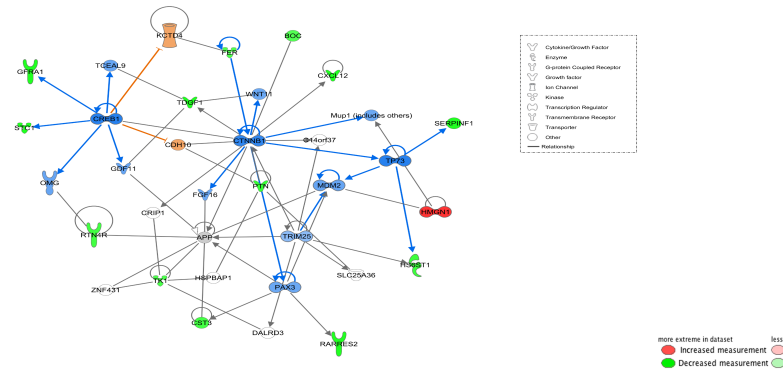
four networks with 13 or more focus molecules, which consisted of those involved in cell death and survival, cell cycle, cancer (p-score=49), cellular movement, organismal injury and abnormalities (p-score=41), cell-to-cell signaling and interaction (p-score=38), free radical scavenging, small molecule biochemistry, and lipid metabolism pathways (p-score=25) (Figure 22B).

Most of the significantly regulated proteins in the embryonic development network were downregulated by influenza infection at 12 hpi. These molecules include CXCL12, RTN4R, BOC, GFRA1, SERPINF1, TDGF1, CST3, FER, PTN, TK1, HS6ST1, RARRES2, and STC1. At 24 hpi, this network appeared with the same trend of downregulation for these proteins, except for BOC, PTN, and CST3, which were either upregulated considerably or not continued their significant downregulation pattern by 24 hpi (Figure 23A). The molecule activity predictor feature of IPA software predicted the potential inhibition or activation of other member molecules in this network that were not significantly modulated or not found in the dataset (Figure 23B). Therefore, using grow tool and based on experimental regulations as well as bioinformatic prediction, the network was connected to different biofunctions related to early differentiation phase of embryonic stem cells and embryogenesis, most significantly including but not limited to development of body trunk, cardiogenesis, development of body axis, proliferation of stem cells, vasculogenesis, proliferation of embryonic cells, growth of embryonic tissue, and differentiation of stem cells. All of these functions were predicted to be inhibited (Figure 23C). Collectively, influenza-induced modulations in protein-protein interaction networks suggest the dysregulations of several proteins that negatively affect the differentiation phase of pluripotent stem cells.

A



B



C

Functions	Prediction	p-value	Molecules
development of body trunk	Inhibited	1.08E-08	CREB1, CRIP1, CST3, CTNNB1, CXCL12, FGF16, GDF11, HS6ST1, MDM2, PAX3, PTN, TDGF1, TK1, TP73, TRIM25, WNT11
cardiogenesis	Inhibited	8.35E-08	CREB1, CRIP1, CTNNB1, CXCL12, FGF16, GDF11, MDM2, PAX3, TDGF1, TP73, WNT11
development of body axis	Inhibited	4.57E-07	APP, CREB1, CST3, CTNNB1, CXCL12, GDF11, HS6ST1, MDM2, RTN4R, SERPINF1, STC1, TDGF1, TP73, WNT11
proliferation of stem cells	Inhibited	9.42E-07	APP, CTNNB1, CXCL12, PAX3, RARRES2, SERPINF1, WNT11
vasculogenesis	Inhibited	1.22E-06	APP, CREB1, CST3, CTNNB1, CXCL12, HS6ST1, PTN, SERPINF1, STC1, TDGF1, TP73, WNT11
proliferation of embryonic cells	Inhibited	2.55E-06	APP, CTNNB1, CXCL12, PTN, RARRES2, SERPINF1, WNT11
growth of embryonic tissue	Inhibited	6.12E-06	APP, CST3, CTNNB1, CXCL12, PTN, RARRES2, WNT11
differentiation of stem cells	Inhibited	6.46E-05	APP, CTNNB1, GFRA1, MDM2, SERPINF1, WNT11

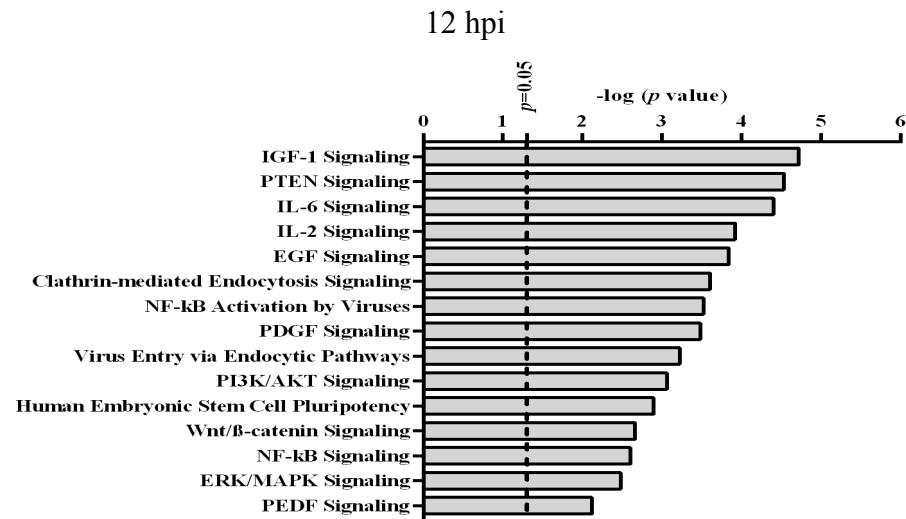
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Figure 23. Bioinformatic analysis of embryonic development network. **A)** Changes in embryonic development network across two time-points. **B)** IPA prediction on the expression patterns of molecules that belong to embryonic development network but not found to be either differentially regulated or identified in my datasets. **C)** Selection of some biofunctions that were predicted to be inhibited based on this network at 24 hpi. These functions are mostly related to early differentiation phase of embryonic stem cells and embryogenesis.

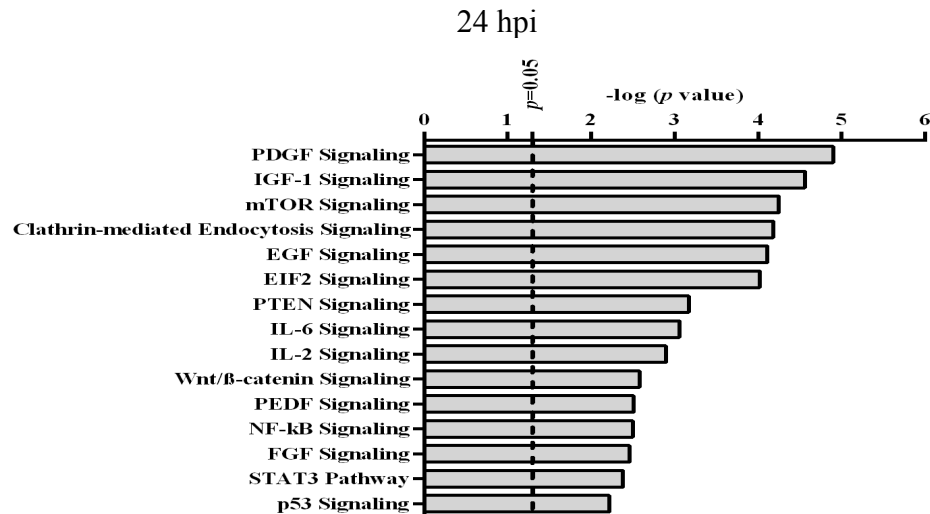
3.8. PR8 infection dysregulates a wide variety of signaling pathways in hiPSCs

Regardless of a total of 494 pathways that underwent changes by significantly differentially expressed proteins at two time points, IPA predicted NF- κ B activation by viruses, virus entry via endocytic pathways, PI3K/AKT signaling, human embryonic stem cell pluripotency, and ERK/MAPK signaling, as the top time-point specific affected canonical pathways by PR8 infection of hiPSCs at 12 hpi (Figure 24A), while top unique influenza-modulated canonical pathways at 24 hpi included mTOR signaling, EIF2 signaling, FGF signaling, STAT3 pathway, and p53 signaling. (Figure 24B). Common top dysregulated pathways across two time-points were IGF-1 signaling, PTEN signaling, IL-6 signaling, IL-2 signaling, EGF signaling, clathrin-mediated endocytosis signaling, PDGF signaling, Wnt/ β -catenin signaling, NF- κ B signaling, and PEDF signaling. Because the affected member molecules from these targeted pathways were not as many as the total numbers of molecules present in those pathways based on SOMAScan screening, IPA software did not predict significant inhibition or activation patterns for the vast majority of influenza-modulated signaling pathways. Most of top affected signaling pathways shown in Figure 24 at both 12 and 24 hpi are known to play critical roles in regulating the differentiation and pluripotency of embryonic stem cells. To understand how considerable the impact of IAV was on the components of a specific pathway, virus-induced dysregulations in member molecules of different signaling pathways were determined by IPA based on the previous studies concerning the expected regulation patterns in these molecules upon activation of a pathway (Figure 25). Due to the involvement of similar member molecules among different targeted pathways, I generated the network of common molecules that are altered significantly by the virus and might individually or collectively

A



B



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Figure 24. IPA prediction of top affected canonical pathways by differentially expressed proteins at 12 hpi (A) and 24 hpi (B). Protein IDs together with their P-values and levels of regulation were uploaded into IPA tool. In the canonical pathway section, p-values of pathways were automatically calculated by the software and top significantly affected pathways were graphed.

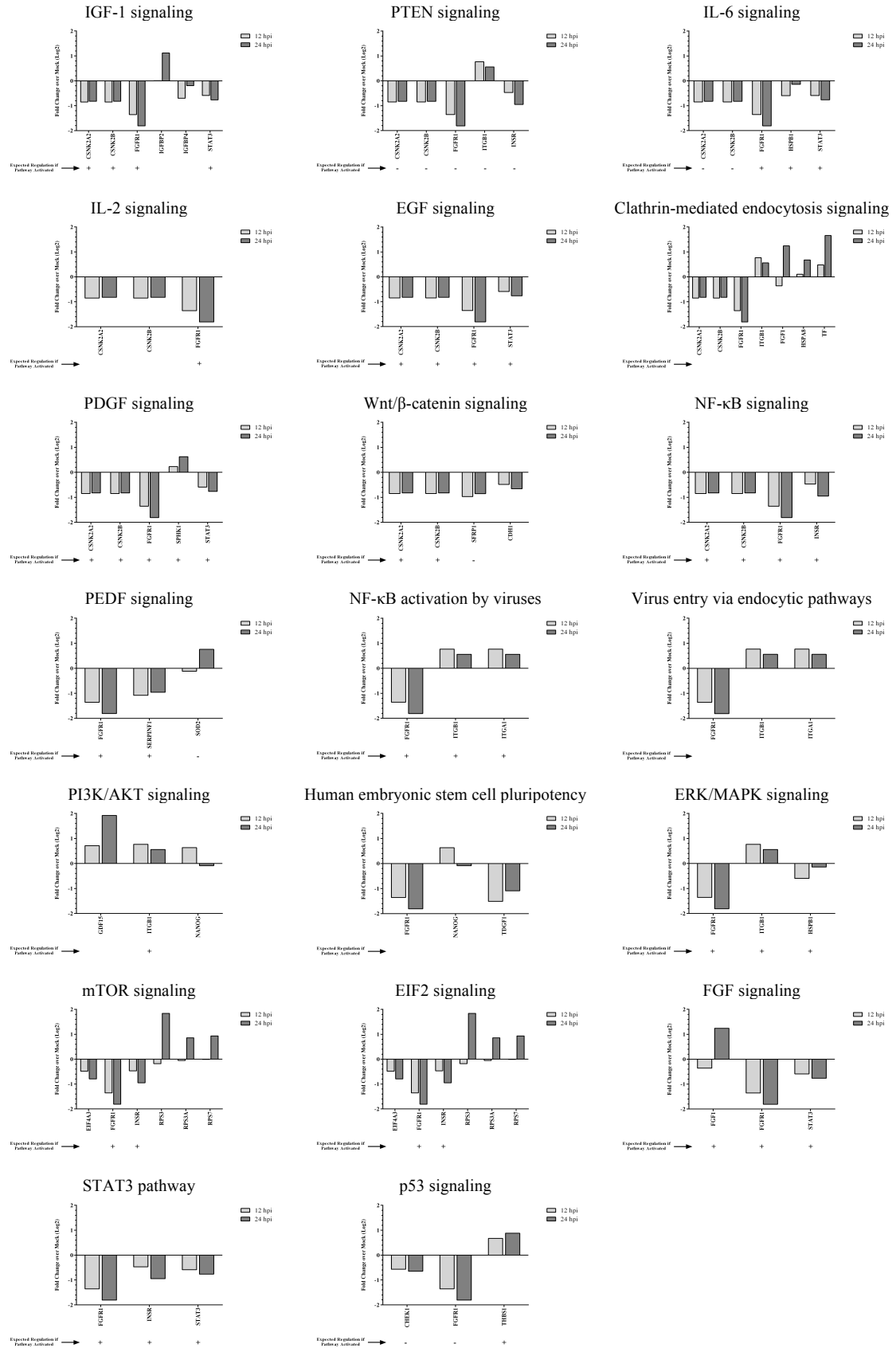
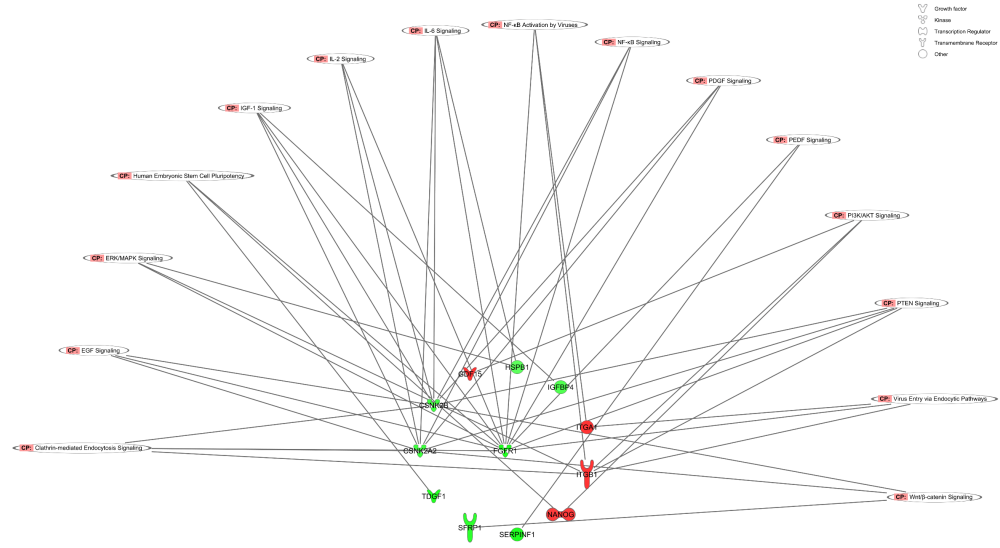


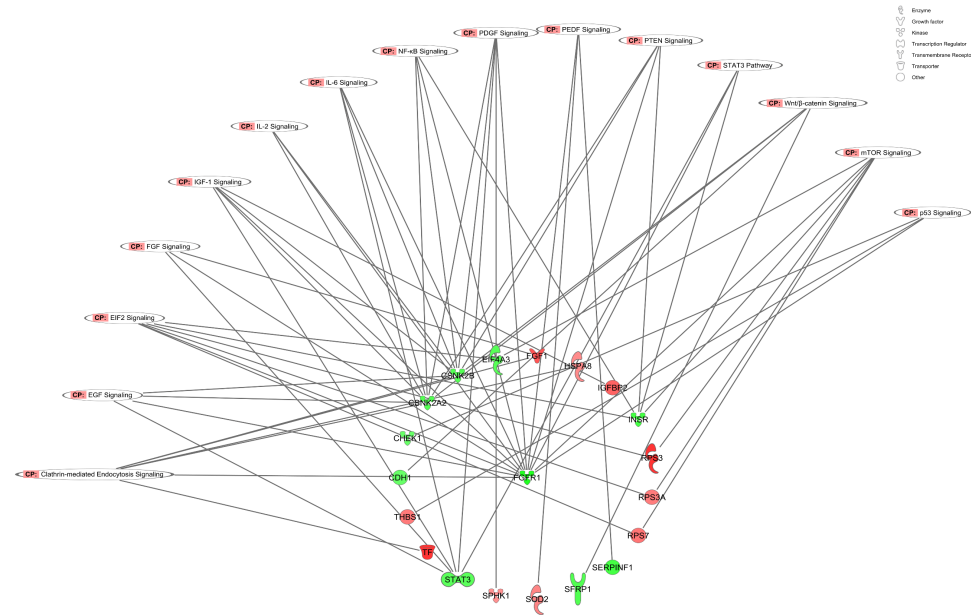
Figure 25. Impact of IAV on the regulation patterns of member molecules from top affected canonical pathways. IAV-modulated modifications in member molecules of various pathways were characterized by IPA based on the former reports on the expected regulation patterns in affected molecules upon activation of a pathway under normal conditions. Published in Cell Death & Disease (Zahedi-Amiri et al. 2019) [160]

mediate virus-induced modifications into top affected pathways. For dysregulated pathways at 12 hpi, these common molecules include SERPINF1, SFRP1, TDGF1, CSNK2A2, CSNK2B, GDF15, HSPB1, IGFBP4, ITGA1, ITGB1, NANOG, and FGFR1 (Figure 26A). For the network at 24 hpi, in addition to CSNK2A2, CSNK2B, SERPINF1, SFRP1, and FGR1, other affected molecules were also found to cause dysregulations in top affected pathways, such as FGF1, HSPA8, IGFBP2, INSR, RPS3, RPS3A, RPS7, SOD2, SPHK1, STAT3, TF, THBS1, CDH1, CHEK1, and EIF4A3 (Figure 26B).

A



B



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Figure 26. Network of common molecules mediating influenza-induced alterations into top affected canonical pathways.

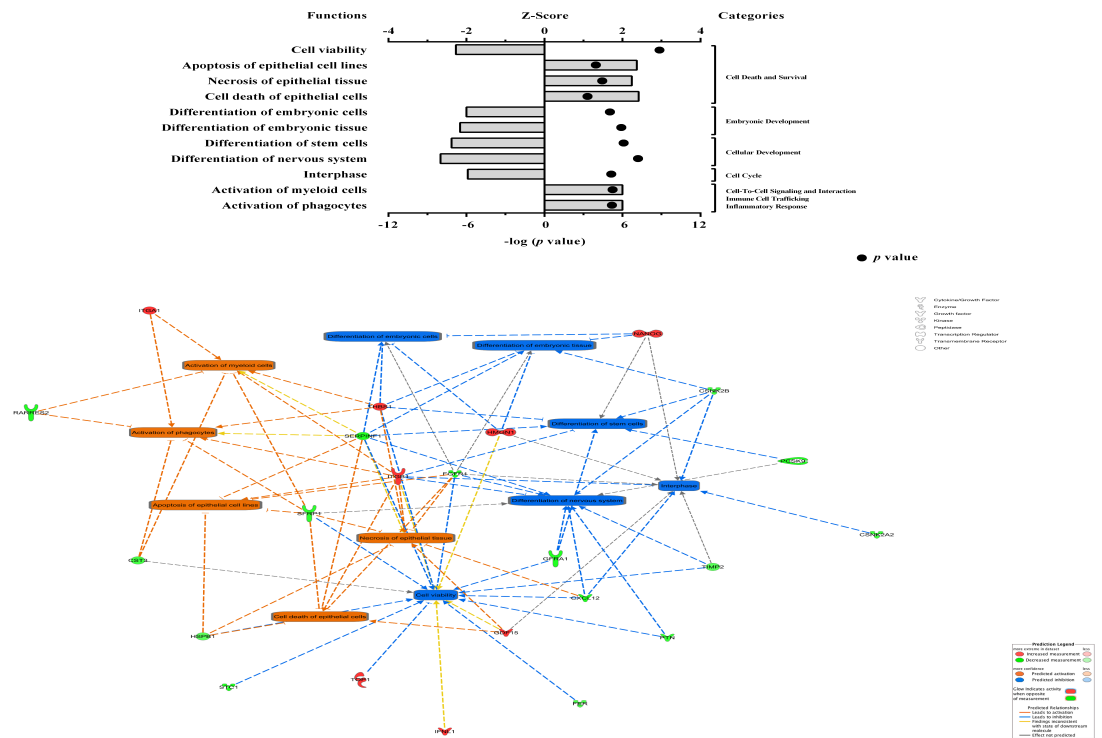
3.9. Inhibited biofunctions by IAV are related to differentiation capacity

This IPA prediction feature also allowed me to determine the potential activation or inhibition of top altered bio-functions and diseases following flu infection in iPSCs. Interestingly, based on z-score of regulated proteins at 12 hpi, the software predicted significant decreases in differentiation of stem cells, embryogenesis and cell viability and significant increases in induction of cell death by necrosis or apoptosis (Figure 27A), emphasising that IAV may lead to cell death and suppression of differentiation in hiPSCs within the first 12 hours of infection. Nevertheless, modifications in a few bio-functions like the formation of epithelial tissue or organization of cytoskeleton and cytoplasm (Figure 27B) might suggest the possibility of abnormal, premature, or spontaneous differentiation in hiPSCs by 24 hrs after PR8 infection.

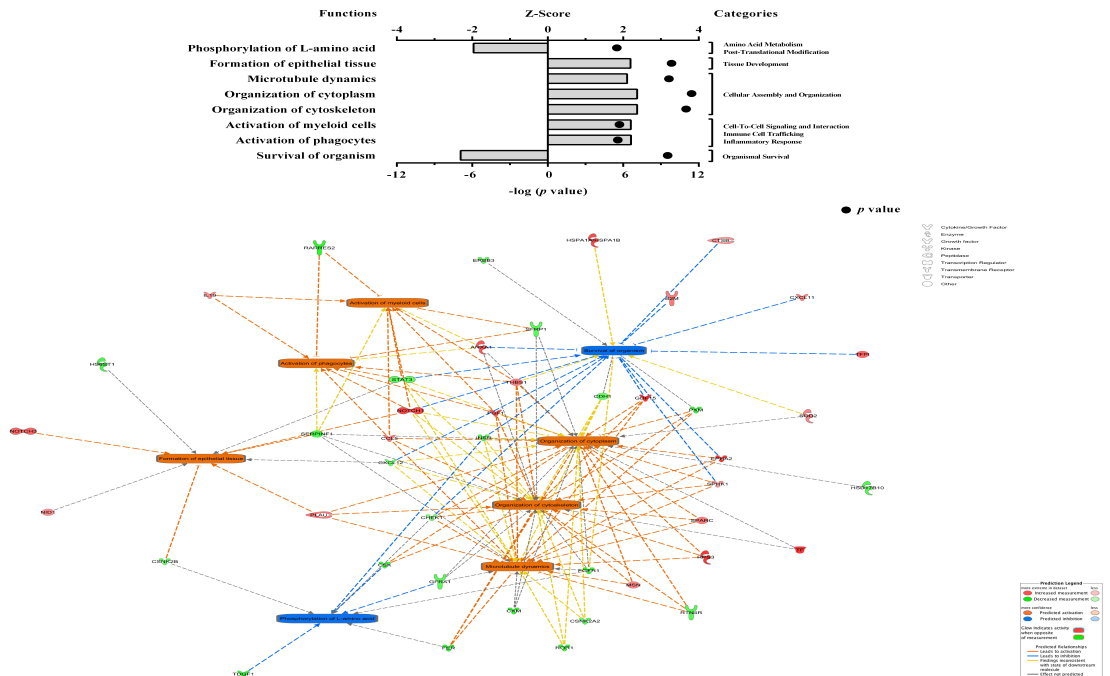
3.10. IAV-triggered autophagy decreases the pluripotency of hiPSCs

Finally, I studied each one of significantly differentially expressed proteins in terms of their relevance and roles in autophagy and pluripotency pathways, according to previous studies, and individually connected them to these pathways based on their regulation patterns and expression at 24 hpi. Some proteins like FGF1, SPARC, STAT3, NOTCH1, FGFR1, and PKM were found to be multifunctional and regulate both autophagy and pluripotency (Figure 28). The expression patterns of these common molecules had reciprocal effects on pluripotency and autophagy, as their specific regulations activate autophagy and inhibit pluripotency at the same time, except for FGFR1, which its down-regulation activate both pathways, suggesting that IAV-induced autophagy decreases the pluripotency by dysregulating a group of proteins involved in controlling both pathways.

A



B



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Figure 27. IPA prediction of top affected biofunctions by differentially expressed proteins at 12 hpi (A) and 24 hpi (B). Protein IDs together with their P-values and levels of regulation were uploaded into IPA tool. In the biofunction sections, activation and inhibition z-scores were automatically calculated by the software and top significantly affected functions were graphed and merged to be displayed as networks. Z-scores values of $\geq 1.96\sigma$ and $\leq -1.96\sigma$ were specified as the criteria for activation and inhibition patterns.

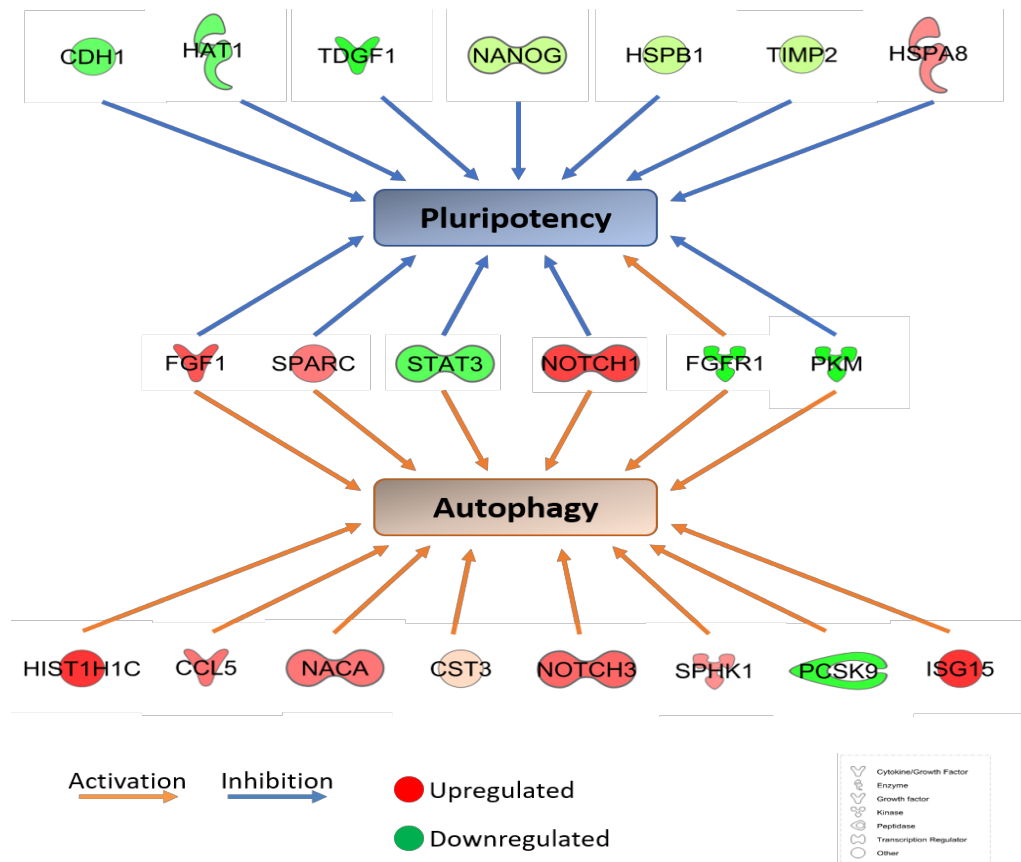


Figure 28. IAV-induced changes in the proteome of hiPSCs affect proteins controlling both autophagy and pluripotency. Differentially expressed proteins were studied individually using data from previous reports regarding their roles in regulating autophagy and pluripotency and connected to pathways based on their regulation patterns at 24 hpi.

Chapter 4. Discussion

Although both mouse and human ESCs either have no, or minor, responses to a broad range of viruses due to the strictly attenuated or absent IFN response pathway, many questions concerning the direct or indirect impact of viruses on the unique characteristics of ESCs like differentiation, pluripotency, and cell death signals have been left unanswered so far. Here, I showed that influenza A virus, as a single-stranded RNA and enveloped virus and one of the inadequately studied pathogens at the embryonic stage, is restricted in propagation in human induced pluripotent stem cells at MOIs of 0.1 and 1 (Figure 6). Wash et al. observed a similar restricted pattern of viral replication in mouse embryonic stem cells (mESCs) after infection with influenza virus A/WSN/33 at MOI of 0.2 [151]. Unlike pluripotent cells, many differentiated somatic cells and some multipotent stem cells support the replication of different IAV subtypes *in vitro*. For instance, subtypes H3N2 (Swine/TX/98) and H1N1 (Human/CA/09) spread productively in swine umbilical cord epithelial stem cells [148]. Similarly, swine lung epithelial stem cells are permissive to various human and animal subtypes, including swine H1N1 (Sw/OH/07), avian H7N2 (Ch/NY/95), and human H1N1 (Hu/OH/06) [149]. Moreover, it has been shown that human neural progenitor stem cells tolerate avian H5N1 (A/Chicken/Thailand/CUK2/04) virus replication to high titers [150].

Apparently, the pluripotency network inhibits viral replication to some extent, and the prominent levels of IAV replication may obey a direct relationship with cellular differentiation and specification. In my study, infection at a higher MOI did not significantly promote PR8 growth by 24 hpi but allowed the immunoblot and immunofluorescence detection of signals for the synthesis of viral proteins, including NS1,

which alone confirms the entry of viral ribonucleoproteins (vRNPs) into the nuclei of hiPSCs, and initiation of transcription and translation (Figure 7). At MOI of 1.0, I could not detect any visible signal for IAV proteins by Western blotting. However, the WSN strain has been shown to produce low levels of viral proteins at this MOI in mESCs by 10 hpi [151].

Depending on the origins, such cell type-specific differences in various pluripotent stem cells may alter the minimum threshold for permissibility to IAV. Compared to another (-)ssRNA virus such as measles, which replicates more in iPSCs [166], the assembly of infectious progeny influenza A viruses appeared to be disrupted in my experiments with iPSCs, even though viral proteins synthesized at higher MOI confirm that the cell's transcription and translation machineries are hijacked by IAV. The MOI-dependent protein production of IAV in PSCs could be interpreted by several unique and intrinsic properties of these cells. For example, it has been speculated that the underdeveloped glycosylation system in pluripotent stem cells hinders viral haemagglutinin (HA) and neuraminidase (NA) maturation [151, 167-170], thereby resulting in not only reduced binding to cell receptors and less fusion with endosomal membrane but also decreased budding of new virions. Additionally, utilizing La Crosse virus (LACV) and Sendai virus (SeV) as other (-)ssRNA viruses to test antiviral responses in ESCs, Wang et al. noticed altered initial MOI, inefficient viral replication and the fast doubling time of mESCs in both control and infected cultures [171]. This rapid proliferation level of pluripotent cells might also have negatively influenced the MOI at time point zero and ultimately the ability of IAV to release measurable numbers of progeny particles from hiPSCs. Growth conditions of these cells can also make barriers in linking the in vitro MOI to a normal in vivo infection. For

instance, since iPSCs are usually grown in colonies on Matrigel-coated surface for improving their attachment and cannot make confluent monolayers, viruses, as charged particles, might be limited in movement toward colonies and could be trapped in the Matrigel to some extent [166, 172], suggesting the potential efficacy of higher MOI for sufficient viral attachment and entry.

I microscopically observed that PR8-infected hiPSCs develop CPE like shrinkage and rounding up in an MOI-dependent manner. The colonies of these infected cells also stained less intensely with crystal violet and demonstrated smaller sizes and fewer numbers (Figure 8). My examination for CPE was consistent with significantly reduced levels of cell viability to less than 30% after a day at MOIs of 0.1 and 5 (Figure 9). Although an MOI of 1 was unable to kill more than 50% of these cells within 24 hrs compared to low and high doses of PR8 infection, the significant decreasing trend of cell viability for all explored MOIs together with visual evidence of CPE collectively suggest the induction of cell death in hiPSCs after exposure to IAV.

Similar cytopathic impacts and loss in cell viability were seen in mESCs following infection with A/WSN/33 strain at various MOIs after one day [151]. The vulnerability and viability of pluripotent stem cells are not equal in response to different DNA and RNA viruses. Likewise, some tested (-)ssRNA viruses have also been shown to develop a diverse array of CPE in these cells. In this regard, Measles replication in hiPSCs leads to loss of stem cell colony mass, enlargement of nuclei, the formation of balloon-like structures, and floating syncytial debris, finally increasing the number of dead cells by 2 dpi [166]. Moreover, the low viral yield of LACV was noted to cause rounding up and detachment of

mESCs from the culture by 48 hpi and diminishes cell viability to <50% at MOIs of 1, 5, and 10 [171, 173].

The multipotent stem/progenitor cells have also been recognized as susceptible as pluripotent stem cells and a variety of differentiated mammalian cells to different subtypes of IAV. For instance, using transmission-electron microscopy, Pringproa and colleagues documented some cytological abnormalities in H5N1-infected neural stem cells, including more cytoplasmic vacuolation, disrupted nuclear membrane, and shrinkage with chromatin condensation [150]. The spectrum of variation and the extent of cytopathic effects that IAV induces are independent of cell potency. Nevertheless, differentiated cells develop IAV-triggered cytopathology later than stem cells. My data indicate that incorporation of RNPs of the PR8 virus into the nucleus alone results in the expansion of cytological deformities and cell death in hiPSCs earlier than somatic cells, despite the restricted replication of this virus under the pluripotent state. These observations may suggest that if IAV infection occurs in early days of pregnancy at the blastocyst stage, embryogenesis could be disrupted in cases of vertical transmission as the ICM is directly affected.

After demonstrating the adverse effects of PR8 infection on cell viability and morphology, I next attempted to discover the type of cell death that was elicited by this strain in pluripotent stem cells. Since the impacts of many viruses on cell death and survival signaling pathways are rarely assessed in these cells, I placed my primary emphasis on apoptosis, the most common type of programmed cell death that can be induced by IAV strains in many differentiated cells through intrinsic and extrinsic mechanisms. The intrinsic apoptotic pathway is activated by stress-mediated mitochondrial damage and can

be regulated by the Bcl-2 family of proteins, which includes both anti-apoptotic and pro-apoptotic molecules [174, 175].

My study shows that PR8 virus is not contributing to intrinsic apoptosis in hiPSCs by 24 hrs, marked by suppressed cleavage of caspases-3, -7, and -9 (Figure 10). These events were accompanied by relatively stable expression of Bax and significant downregulation of Bcl-2, the pro-apoptotic and anti-apoptotic proteins, respectively. The accumulation of p53, an apoptotic regulator, was increased transiently at 12 hpi (Figure 11). I examined the expression of caspases as well as apoptotic regulators in PR8-infected lung epithelial A549 cells and noticed cleavage of caspases and the progressive ratio of Bax/Bcl2 even with decreased levels at 24 hpi, suggesting the induction of intrinsic apoptosis in these differentiated cells. Such a cell-specific difference confirms the unique expression profiles of apoptotic executioners and regulators in iPSCs and the possible contribution of an alternative pathway that leads to cell death in these cells after IAV infection. In somatic cells, apoptosis is known to be temporarily blocked by IAV NS1 protein at preliminary stages of infection via PI3k-Akt signaling pathway for expanding viral replication [32, 176, 177]. This early inhibition of apoptosis then is accompanied by apoptotic or necrotic pathways at subsequent phases of infection to promote the death of infected cells and the release of progeny viruses [178]. In my study, it might be possible that intrinsic apoptotic response was activated at later post-infection time points (e.g., after 24 hpi), which I did not investigate here because of severely decreased cell viability by 24 hpi. Therefore, I hypothesize that observed cell death in pluripotent cells during the first day of exposure to the PR8 virus has occurred in a caspase-independent manner (e.g., by extrinsic apoptosis or another cell death pathway).

The acidification of endosome-lysosome system is needed for proper function of lysosomal enzyme and fusion of autophagosome with the lysosome to complete autophagy, the pathway controlling the degradation of dysfunctional organelles and recycling of cellular components [179]. This may also provide an optimal condition for uncoating IAV particles and the release of RNPs [178, 180, 181]. Interestingly, it has been already reported that IAV induces autophagy in a variety of differentiated mammalian cells [154, 161, 182]. Considering the possibility of cell death with autophagy that happens without chromatin condensation but is associated with extensive autophagic vacuolization of the cytoplasm [183], I detected significant activation of autophagy in both influenza-infected A549 and hiPS cells, noted by the increased accumulation of LC3 β -II and Atg5 together with considerable degradation of the autophagy receptor p62 (Figure 12).

To my knowledge, this is the first evidence of virus-induced autophagy in pluripotent stem cells, despite the lack of proof to directly link this highly activated mechanism to demonstrated cell death. After fertilization by the spermatozoa, autophagy levels were found to be instantly upregulated in murine oocytes [184]. Subsequently, autophagy is inhibited between the 1- and 2-cell stage but can be increased again at the 4-cell stage [185]. However, this mechanism is not clearly understood under pluripotent state and seemed to be stable and dispensable in ESCs residing in the inner-cell mass of the blastocyst. Based on knockout models of autophagy-related genes (ATGs) *in vivo* and *in vitro*, consequences of unnaturally suppressed autophagy in pluripotent cells can be evolved during embryogenesis or later in terminally differentiated cells and may include neuronal inclusions, reduced adipose mass, unwanted accumulation of damaged organelles, and defective embryoid body formation in culture with insufficient ATP

production [186, 187]. In contrast, implications of excessive-abnormally induced autophagy (e.g., by the virus like in the present study) may manifest subtlety in the undifferentiated pluripotent stem as well as in derived tissues and yet remained to be identified.

Some believe the ubiquitin-proteasome system (UPS) may compensate for the intrinsically low level of autophagy in the pluripotent state, due to high proteasome activity in these undifferentiated cells. Conversely, polyubiquitinated proteins are highly expressed in differentiated cells, and proteasome activity is progressively inhibited during the differentiation process [188]. Furthermore, higher levels of autophagy were naturally observed during the differentiation of ESCs, probably through degrading protein complexes that regulate the pluripotency [189]. Not surprisingly, many viruses develop mechanisms to evade, subvert, or exploit cellular processes for their benefit. I speculate that the PR8 virus induces autophagy in hiPSCs differently from its normal upregulation upon certain conditions. Such an altered viral-triggered mechanism under pluripotency conditions suggests the possible initiation of the upcoming premature or abnormal spontaneous differentiation in IAV-infected pluripotent stem cells, as elevated levels of autophagic activity were seen in differentiating PSCs. It is thus necessary to evaluate the level of pluripotency and the normal germ layers differentiation capacities in these pluripotent cells after IAV infection to decipher the characteristics of influenza-induced autophagy and its role in probable abnormal differentiation. Hypothetically, with regards to demonstrated cell death, the virus may simultaneously put iPSCs under differentiation and promote autophagy or may keep them in an undifferentiated state while increasing autophagic activity. It is also possible that the expected differentiation is not directly

modulated by the virus itself and could be the outcome of virus-induced excessive autophagy. In all cases, cell death could be executed via apoptotic or non-apoptotic pathways and whether autophagy is actually playing a role in the cellular demise of hiPSCs remains unclear [183].

The effects of viral replication or entry on pluripotency have not yet been described for most viruses, including IAV. Moreover, specifying the level of pluripotency gives valuable information about the possibility of premature spontaneous differentiation in virus-infected iPSCs. I found influenza-infected hiPSCs to exhibit a remarkable decrease in the expression of pluripotency regulators Nanog, Sox2, and Oct-4A by 24 hpi (Figure 13). This is the first evidence so far that proves PR8 infection can negatively affect the pluripotency network of hiPSCs. Measles, as the only other (-)ssRNA virus tested for its impact on pluripotency, was seen to be incapable of altering this intrinsic feature of these cells, but blocks the directed differentiation of infected iPSCs to ectoderm, endoderm, and mesoderm layers because of causing extensive cytopathic effects like formation of syncytium and balloon-like structures [166]. Significant levels of cell death that happened by 24 hpi and necessity of using higher MOI did not allow me to perform functional confirmation by embryoid body assay for testing the capability of PR8-infected cells for further differentiation into the three germ layers.

Nevertheless, reduced pluripotency noted in the current study together with my findings regarding IAV-induced autophagy suggests a link between excessive autophagy and loss of pluripotency, which may contribute to potential premature or abnormal spontaneous differentiation. Some studies suggest that mTOR acts as the regulator of pluripotency and self-renewal in hESCs, in addition to its central role in the regulation of

autophagy pathway and triggering several downstream pathways including PI3-kinase/Akt and AMP-activated protein kinase [185, 190, 191]. Zhou and co-workers reported a set of outcomes in hESCs after inhibition or depletion of mTOR, including considerable reduction in the expression of Oct4 and Sox2, elevated mesoderm and endoderm differentiation, and limited proliferation [192]. At 24 hpi, I obtained similar results on the declined expression of Oct4 and Sox2 after influenza-induced autophagy. Additionally, my SOMAScan proteomic screening revealed evidence of potentially abnormal differentiation based on dysregulated proteins at the same post-infection time point. In A549 cells, mTOR inhibition with rapamycin is known to increase autophagic activity as well as the production of viral NP and NS1 proteins but reduces the number of infectious progeny viruses [178]. I also pharmacologically inhibited mTOR using Rapamycin and noticed significant loss of pluripotency, higher level of viral growth and protein production, and elevated levels of autophagic activity (Figures 14 and 15). In my study, PR8-infected hiPSCs showed not only significant levels of viral protein production but also restricted viral replication and caspase-independent or non-apoptotic cell death under excessive autophagic activity.

Irrespective of my previous assumptions about the type of cell death and possible reasons of limited viral replication, it has also been hypothesized that mTOR suppression stimulates upstream receptor tyrosine kinase signaling, subsequently activating the Akt/PKB pathway, which may inhibit apoptosis. According to a transcriptomic analysis, mTOR is upregulated during differentiation and afterward compared to its expression under the pluripotent state of hESCs [193]. In this study, I did not directly evaluate the expression of mTOR in IAV-infected hiPSCs. However, my SOMAScan proteomic screen

showed that some mTOR signaling molecules like FGFR1 and INSR1, that are expected to be up-regulated upon pathway activation, were significantly down-regulated by 24 hpi, suggesting potential IAV-triggered inhibition of this pathway (Figure 25). Hence, my results, and those of others suggest that PR8 virus promotes autophagy in these pluripotent stem cells, possibly by down-regulation of mTOR, which can decrease the level of pluripotency, ultimately inducing premature or abnormal spontaneous differentiation. Further investigations are required to explain the initiation of influenza-induced autophagy in pluripotent cells and its possible impact on the differentiation into germ layers.

Following the obvious influenza-induced changes in cell viability and pluripotency, proteomic screening was performed on 1,307 proteins using SOMAScan assay to find out how much of the examined proteome is dysregulated by the virus and its associated alterations. My statistical analysis showed that infecting hiPSCs with the PR8 virus resulted in significant changes only in 2.4% and 5.3% of the assessed proteome within 12 and 24 hpi, respectively (Figures 16 and 17).

This observation is consistent with what has been previously reported about the limited responses of these cells to a broad range of viruses [152]. Moreover, mESCs had also indicated minor dysregulations at the transcriptomic level after 6 hrs of infection with influenza A/WSN/33 [151]. Such limited host responses to influenza infection might be because of inhibited viral replication in these pluripotent cell populations. At 12 hpi, although a few proteins like Interleukin-29 (IL-29/IFNL1) and Interferon-stimulated gene 15 (ISG15), that are involved in type 3 and 1 interferon responses, were upregulated to less than two-fold, an apparent deficiency was noticed in the expression of proteins related to innate immune responses against viral infection (Table 1). Inactivated immune responses

were already seen in pluripotent stem cells after different viral or bacterial infections and are mainly assumed to be due to reciprocal inhibition between the IFN system and pluripotent state in these cells, which evokes RNA interference mechanism to compensate for the underdeveloped innate immunity in PSCs and may potentially suppress viral replication through RNA silencing complex and cleavage of viral RNA [152]. It is also believed that epigenetic shutdown of foreign genes like viral mRNA via TRIM21-mediated cascade inhibits viral production in these cells [152, 194, 195]. Highly conserved regulation of transcription in ESCs can be considered as another condition that may downregulate interferon-stimulated genes for maintaining pluripotency [151, 196].

The IPA analysis of differentially regulated proteins at 12 hpi also highlighted significant negative impacts on the activation of some canonical pathways controlling immune responses, including leukocyte extravasation signaling, IL-2 signaling, and IL-6 signaling (Figure 24A). It is possible that the 1.5-fold upregulation of ISG15 at this time point can be partially explained by the intrinsic immature immunity of pluripotent stem cells to this virus, as it has been shown that this protein can ISGylate (protein conjugation-mediated anti viral activity) IAV NS1 protein which leads to the inefficiency of NS1 and limited viral replication, the same situation I observed experimentally. IPA software predicted some potentially affected biofunctions as well, and these data collectively support the induction of cell death by either necrosis or apoptosis, reduction of cell viability, and inhibition of differentiation in ESCs (Figure 27A).

I found these bioinformatic predictions reliable and consistent with what I have already acquired on induction of cell death and stable pluripotency at 12 hpi. Even though I did not show the normal expression of pluripotency markers by 12 hpi through other assays, my

proteomic screening determined the upregulation of NANOG, thus confirming the pluripotency of PR8-infected hiPSCs and repressed differentiation at the mentioned time point. In contrast, at 24 hpi, IPA detected dysregulations among some top affected canonical pathways that are involved in processing the normal differentiation into germ layers and embryogenesis, such as PDGF signaling, IGF-1 signaling, and EGF signaling (Figure 24B). Dysregulated proteins found in such pathways can also be involved in controlling several other cellular pathways that are not directly connected to stem cells characteristics and differentiation. In addition, IPA predicted the activation of a few biofunctions like cell movement, the formation of epithelial tissue, and organization of cytoplasm/cytoskeleton, which are often seen during the differentiation phase of ESCs. This may support the idea that cells are already committed to specific fates and spontaneous differentiation within 24 hpi. However, these changes appeared to be highly dysregulated by IAV infection and can influence the capacity of PSCs for normal differentiation toward the formation of germ layers. More studies should be carried out to test the effects of such IAV-induced changes on signaling pathways that direct the differentiation of pluripotent stem cells into ectoderm, endoderm, and mesoderm layers.

Conclusion

In conclusion, despite restricted viral replication and limited levels of caspase-dependent intrinsic apoptosis, my results indicated IAV causes cytopathology in hiPS cells and can reduce their viability and pluripotency. For the first time, it was also noticed that such an IAV-triggered decline in pluripotency is associated with an excessive level of autophagy, possibly through inhibiting mTOR, which ultimately may result in spontaneous

differentiation of pluripotent stem cells. Moreover, my SOMAScan proteomic screening suggests dysregulations in several signaling pathways regulating differentiation in these cells within 24 hrs after influenza infection. These findings can be useful for modeling the intra-blastocyst infection with influenza A virus and characterizing the potential effects of this pathogen on early stages of embryogenesis.

Future perspectives

Further studies are needed to discover the molecular mechanisms involved in the loss of pluripotency, initiation of influenza-induced autophagy in pluripotent cells, and possible effects of these phenomenons on signaling pathways controlling differentiation into three germ layer cell populations. For instance, in order to confirm that hiPSCs actually lost their pluripotency, a functional confirmation could be done by doing an embryoid body assay with a large amount of infected stem cells to quantify the expression of endoderm, ectoderm and mesoderm markers at different timepoints and MOIs for comparing with mock-infected controls. This approach could be valuable in understanding that the differentiation into what germ layer is affected most by the virus. IAV-modulated alterations in the proteome of pluripotent stem cells can also be assessed within differentiation into a certain germ layer. Moreover, given that excessive levels of autophagy in infected hiPSCs was induced by IAV based on my assumptions, it is also possible to establish a causal association between autophagy induction and loss of pluripotency by performing additional experiments utilizing autophagy inhibitors and inducers for testing both the infectivity of the virus and the level of pluripotency. The mechanism behind IAV-induced autophagy in pluripotent stem cells can be further

confirmed by investigating mTOR inhibition at both proteomic and transcriptomic levels. Concerning the restricted IAV replication, it would be interesting to measure the expression of viral mRNA for looking at potential epigenetic shutdown of some viral genes that are involved in the maturation and release of the progeny viruses. Additionally, I used PR8 virus in this study, which is a lab adapted and attenuated strain from subtype H1N1. To characterize the strain- and subtype-specific differences, most of experiments can be reproduced with other IAV subtypes and strains.

Another possibility for studying the embryonic origins of influenza-mediated congenital malformations in offspring is to differentiate pluripotent stem cells into various multipotent stem cell populations that form terminally differentiated tissues with hypothetical defects. Depending on targeted tissue, infection with IAV can be triggered either before or after inducing differentiation into multipotent progenitors to develop a 3D *in vitro* model for determining potential virus-induced dysfunctions and malformations through conducting several histological, molecular, and physiological assessments. Last, but not least, modeling both intranasal and *in vitro* fertilisation (IVF)-administered infection with influenza in vaccinated and non-vaccinated animals would not only help in understanding the possibility and mechanism of transplacental passage of the IAV, but may also shed light on the role of this virus in the development of congenital anomalies with embryonic origins.

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