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Human Induced Pluripotent Stem Cell Based Cell Assays; an improvement in disease modeling and toxicity testing?

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Abstract

Human induced Pluripotent Stem Cell (hiPSC) based cell assays are a new promising technology which can help unravel complex interactions and by this cutting in the time and money spend on disease modeling and toxicity testing. HiPSC are somatic cells converted to pluripotent stem cells through expression of a small combination of transcription factors raising the possibility of producing custom-tailored cells for the study of numerous diseases. Indeed, hiPSCs have already been derived from patients suffering from a large variety of disorders indicating the potential of hiPSC. HiPSC possess as the name implies the possibility to differentiate into all cell types of the human body. In this review we will discuss the advantages and limitations of hiPSC based cell assays and the advantages and limitations of the current methods of disease modeling and toxicity testing by research animals and immortalized cell based assays. After that the conclusion about the use of hiPSC based cell assays in disease modeling and toxicity testing is given.

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Index

Page 1	Cover page
Page 2	Abstract
Page 3	Index
Page 4-6	Introduction
Page 7-9	Disease modeling by hiPSC
Page 10-11	Toxicity testing by hiPSC
Page 12	Conclusion
Page 13-14	References

Introduction

The current method of disease modeling and toxicity testing is by using immortalized cell lines and research animals, however, both of these methods have their drawbacks. The immortalized cell lines are genetically altered, aneuploid and may therefore cause irrelevant responses to compounds (Anson et al., 2011). Drawbacks of the use of research animals are numerous. First, cells isolated from animal tissue lose their in vivo phenotype, second cells isolated from animal tissue may exhibit high variability from one isolation to the next, third differences between species will influence the results and finally there is widespread disaffection in the community for the use of animals in research (Anson et al., 2011). The idea is that human induced Pluripotent Stem Cell (hiPSC) based cell assays could be a good alternative for immortalized cell lines and research animals in disease modeling and toxicity testing. In this article first some general information about hiPSC is given, secondly disease modeling in diabetes mellitus will be handled, after that toxicity testing and finally the conclusion on hiPSC cell based assay as an option in disease modeling and toxicity testing will be given.

It is known since the 1960's that in amphibians the transfer of the nucleus of keratinocytes, a differentiated adult somatic cell type in the arrested G0 growth phase, to the respective unfertilized enucleated oocyte, results in embryos that develop normal to the juvenile tadpole stage with the characteristic phenotype of the keratinocyte donor (Wilmut, Schnieke, McWhir, Kind, & Campbell, 1997). The transfer of the keratinocyte nucleus to the enucleated oocyte and the subsequent dedifferentiation of the Xenopus keratinocyte nucleus revealed for the first time that it was possible to dedifferentiate a differentiated adult somatic cell. This process came known in the general population as cloning. This dedifferentiation of the keratinocyte nucleus has to be done by factors present in the enucleated unfertilized oocyte since that is the only source possible. This means that Xenopus ESC can be generated by a cocktail of certain factors present in the enucleated unfertilized oocyte. However, since Xenopus only reaches the juvenile stage it leaves the question open if a differentiated nucleus can be fully reprogrammed so that it develops into all tissues of an adult Xenopus (Wilmut et al., 1997).

In 1996 the first mammalian sheep derived fibroblast cell nucleus was transferred to the respective unfertilized enucleated oocyte with the viable offspring Dolly as a result (Wilmut et al., 1997). This oocyte was recovered from the sheep 28-33 h after gonadotropin releasing hormone treatment, and enucleated as soon as possible (Wilmut et al., 1997). The oocytes were recovered in calcium and magnesium free Phosphate Buffered Solution containing 1% FCS and transferred to calcium free M2 medium containing 10% FCS at 37C (Wilmut et al., 1997). After recovery the cells were made quiescent and diploid by reducing FCS from 10% to 0,5% FCS in 5 days forcing the cells to exit the cell cycle and go to the G0 arrested phase (Wilmut et al., 1997). The enucleated oocyte was fused with the keratinocyte by an electrical pulse, this pulse also started the growth of the cell again (Wilmut et al., 1997). After the pulse the fused enucleated oocyte was implanted in the sheep (Wilmut et al., 1997). This sheep clone revealed for the first time that in mammals it was also possible for a differentiated fibroblasts nucleus to be dedifferentiated by undefined factors present in the respective enucleated oocyte (Wilmut et al., 1997). Dolly also revealed for the first time that a clone could reach adulthood indicating that a dedifferentiated cell can become all tissues of the clone (Wilmut et al., 1997).

In 1998 the isolation of human Embryonic Stem Cells (hESCs) was achieved for the first time (Thomson et al., 1998). This isolation of hESCs opened new therapeutic solutions in humans and the research in ESCs grew exponentially (Hussein, Nagy, & Nagy, 2011).

However, the use of ESCs in medicine faces major difficulties to overcome namely, immune rejection and the necessity to use early stage embryos for ESC isolation (Hussein et al., 2011).

In 2006 a groundbreaking discovery was done by Takahashi and Yamanaka who revealed that enforced expression of a defined set of transcription factors known as the Yamanaka factors: Klf4, Oct4, Sox2, and c-Myc could revert the phenotype of differentiated adult somatic cells back to generate ESC like pluripotent cells, termed induced pluripotent stem cells (Takahashi & Yamanaka, 2006). This discovery was based on the hypothesis that genes who help maintaining ES cell identity also play a role in pluripotency induction (Takahashi & Yamanaka, 2006). This hypothesis was tested and in this pioneering study 24 different candidate transcription factors were retrovirally transduced into mouse fibroblasts. These original 24 transcription factor were chosen for their inductive capacities in adult somatic cells (Takahashi & Yamanaka, 2006). The 24 transcription factor cocktail was later further narrowed down to just the four Yamanaka factors (Takahashi & Yamanaka, 2006). In November 2007 Yamanaka showed in "Cell" that it was also possible to generate human induced pluripotent stem cells (hiPSC) (Takahashi et al., 2007). Since then the generation of specific functional cell types from hiPSCs has been demonstrated, including neural cells, vascular epithelial, smooth muscle cells, cardiomyocytes, hematopoietic cells, pancreatic insulin producing cells, and hepatocyte like cells amongst other types of somatic cells (Inoue & Yamanaka, 2011).

iPSCs can with the current technology be derived by different combinations of transcription factors and small molecules, introduced in the cell by a variety of methods. These methods include retroviral, lentiviral, transposon, ΦC31 phage integrase, sendai virus, plasmid based, episomal plasmids, protein, minicircle vector and mRNA for insertion of the transcription factors (Anson et al., 2011). In this article the focus is on the information derived from experiments performed with the original reprogramming cocktail of the four transcription factors found by Takahashi and Yamanaka. The standard procedure for the generation of iPSC is to isolate and culture the donor cells. Second transfect the Yamanaka factors by viral vectors. Third harvest and culture the cells under ESC conditions using mitotically inactivated feeder cells. Finally a small subset of cells will develop to iPSC and develop into ESC like colonies, which will be picked and expanded see figure 1.

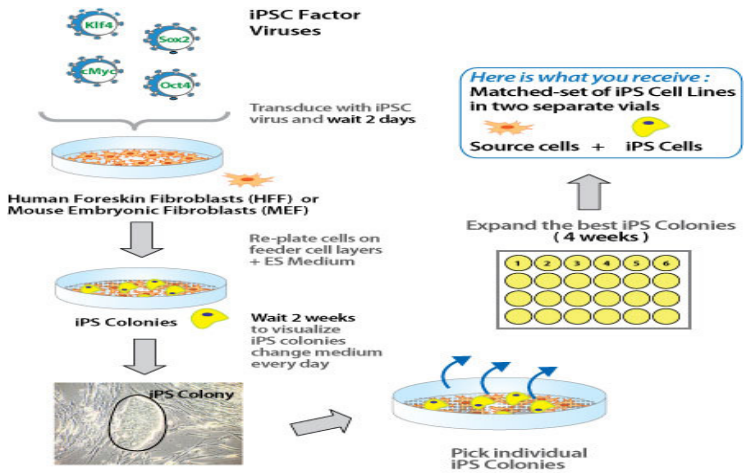

Figure 1 Generating iPSC with the four Yamanaka factors. Step 1 transduce with iPSC viruses and wait 2 days. Step 2 Re-plate cells on feeder cells + ES medium. Step 3 Wait 2 weeks to visualize iPSC colonies and change medium every day. Step 4 pick individual colonies and expand the best ones (4 weeks) (biocat.com)

However, how to validate iPSC, the best method herefore is by doing four assays. see figure 2.

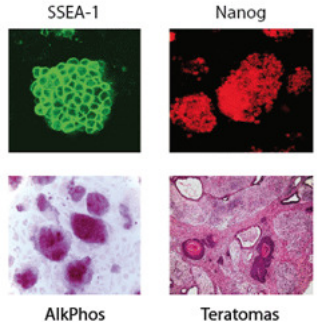

Figure 2. The detection of the stem cell markers SSEA-1 and Nanog by immunocytochemistry, the confirmation of alkaline phosphatase activity using an AP Detection Kit and the performance of teratoma assays by subcutaneous injection of iPS cells into the dorsal flank of SCID mice. Teratomas were visualized after 4 weeks with hematoxylin and eosin staining.

Although generating iPSC with the four Yamanaka transcription factors has become a standard procedure, the mechanism by which these four factors induce pluripotency just starts to be unraveled.

It is now believed that the inefficiency of the reprogramming process in iPSC induction is caused by epigenetic barriers that are only overcome incidental (Plath & Lowry, 2011). Recent data also indicate that these repressive chromatin states are a major physical barrier to the induction of pluripotency in normal differentiated cells (Plath & Lowry, 2011). Because multiple different signals can act on the repressive chromatin states, these signals can work alone or in concert to change reprogramming and these signals can even affect the activity of the reprogramming factors. This demonstrates the close relationship of different pathways in regulating reprogramming to the iPSC state (Plath & Lowry, 2011). The finding that iPSCs carry an epigenetic memory of the starting cell may shed light on the reason why some processes are so difficult to reset during reprogramming (Plath & Lowry, 2011). This finding also shows that the choice of starting cells for hiPSC based cell assays is very important (Plath & Lowry, 2011). The hypothesis that only non-lineage committed cells or adult stem cells are possible to reprogram has also been discarded as an explanation for the low efficiency based on the ability of terminally differentiated cells, such as pancreatic islets or terminal blood lineages, to give rise to iPSCs (Plath & Lowry, 2011).

These findings have led to a model which proposes that expression of the reprogramming factors alone is not enough to allow the change to pluripotency (Plath & Lowry, 2011). Additional signals that act on the repressive chromatin states are required to overcome the major epigenetic barriers that prevent the reprogramming of adult differentiated somatic cells from occurring (Plath & Lowry, 2011).

The next big technological leap is to directly create the desired cell type. This is done by the use of tissue and lineage specific promoters so that the somatic cell can directly be reprogrammed into the cell of choice making the process much more efficient (Anson et al., 2011).

Disease modeling by hiPSC based cell assays.

Diabetes mellitus is a common disease in the elderly western population. The occurrence of diabetes mellitus is rising and is estimated by the World Health Organization to cause 5% of all deaths each year worldwide and the number of deaths is estimated to double between 2005 and 2030 (Maehr et al., 2009). This makes diabetes a major cost to society and the health care system and an enormous burden for patients themselves. Diabetes is in general classified into two major categories: type 2 diabetes mellitus and type 1 diabetes mellitus. Type 2 diabetes mellitus is characterized by insulin resistance, and type 1 diabetes mellitus is characterized by a failure of the β cells to produce insulin. In general, type 1 diabetes mellitus is due to the autoimmune destruction of the insulin producing pancreatic β cells. Type 2 diabetes mellitus is due to constant high levels of insulin making cells unresponsive to circulating insulin in the extracellular space. The loss of glycemic control in diabetes mellitus patients leads to complications of the vasculature because the high glucose levels damage the blood vessels by glycosylering. This glycosylering makes diabetes mellitus patients susceptible to complications with the vasculature. Administration of exogenous insulin allows glycemic control by lowering average glucose levels to some extent in patients with T1D however, in type 2 diabetes this only works with a few patients. However, the glycemic control is usually not perfect and may be accompanied by dangerous states of hypoglycemia which could result in coma and finally loss of life. Therefore, administration of exogenous insulin, which is an effective treatment, however, it is not a cure. To cure diabetes mellitus the disease mechanism has to be understood so a new molecular target for diabetes mellitus treatment can be found.

The advantage of disease modeling in amongst others diabetes mellitus research is that it will increase our understanding of disease progression and disease biology in specific cell types (Inoue & Yamanaka, 2011). This increase in knowledge about diabetes mellitus progression and biology can be used in the search for new molecular targets for drug development (Inoue & Yamanaka, 2011).

hiPSC based cell assays are a powerful method used in molecular biology for testing and or measuring the biological activity of a compound in an organic sample. This testing and or measuring on hiPSC based cell assays is done by a method called high throughput therapeutic screening. High throughput therapeutic screening is as the name implies a fast screening method to test many compounds for the one that produces, in the case of diabetes mellitus research, the therapeutic insulin response to glucose in hiPSC differentiated cell based assays (Anson et al., 2011). High throughput screening is the main reason why hiPSC are so useful in disease modeling.

However, what is the advantage hiPSC based cell assays have over the current method of disease modeling by genetically altered cells and or research animals. First of all, because phenotypical and genetical information is obtained, the evaluation of cell assays becomes easier and more informative because an extra level of research is added (Anson et al., 2011). Second, hiPSCs can be generated from patients irrespective of whether the disease is in the familial or the sporadic form. Because of this the disease can be studied from several angles by different hiPSC based cell assays derived from different forms of the disease by example type 1 diabetes versus type 2 diabetes (Anson et al., 2011). Thirdly the ease of deriving tissues such as skin, peripheral blood, and other somatic tissues for hiPSC directly from patient samples enables creation of disease specific hiPSC based cell assays derived from individual patients. From this a large databases of hiPSC useful for disease modeling is created with much ease (Anson et al., 2011). These disease specific hiPSC based cell assays provide powerful *in vitro* disease models for disease modeling by evaluating the differences between models (Anson et al., 2011). The potential of patient specific hiPSC based cell assays also

includes that it could be used to identify patients who would respond adversely or favorably to a drug (Anson et al., 2011). This approach opens powerful new options in personalized medicine (Anson et al., 2011). Fourth because hiPSC can be differentiated into different tissues disease modeling can be studied on different types of cells, making disease modeling from multiple angles possible (Anson et al., 2011). Finally, although hiPSC have genetic artifacts from the Yamanaka transcription cocktail, they are still genetically more intact then the currently used cancer derived immortalized genetically altered cell lines (Anson et al., 2011).
However, hiPSC also has a drawback which is the relative maturity of the hiPSC derived cell lineage, given that most cell types maintain a more embryonic phenotype in culture making the interpretation of results harder and lowering the potential of the use of hiPSC (Anson et al., 2011).

Several human embryonic stem (hESC) based cell assays have been generated and are still considered the standard in pluripotent stem cells based cell assays. In recent years however, pluripotent stem cells have become available by generating hiPSC from somatic cells by the transcription factors found by Yamanka (Maehr, 2011). The first study to try to create hiPSC based cell assay was the generation of hiPSCs from patients with the disease amyotrophic lateral sclerosis. This is a neurodegenerative disease that causes loss of motor neurons in the spinal cord and motor cortex. Although the motor neurons generated in vitro did not show a diseased phenotype, this study is still considered a proof of principle (Dimos et al., 2008). These hiPSC from a patient with amyotrophic lateral sclerosis indicated for the first time the possible potential of hiPSCs as a disease modeling system. Another hallmark of this study was the generation of hiPSCs from relatively old patients (Dimos et al., 2008). This feature will undoubtedly be of major importance in future clinical applications since the general population is getting older and older and diseases of aging are getting more costly for society every year (Hussein et al., 2011). So far, hiPSC based cell assays have been generated from patients who had a variety of diseases; these include a Pima Indian with diabetes and two individuals with autoimmune diabetes (Maehr, 2011). For other human disease specific cell lines see figure 3

Disease category	Disease (Reference)	Differentiation of functional cells	Disease modeling
Neurological	ALS (6)	Yes / motor neurons	No
	PD (5, 12)	No(5), Yes(12) / dopaminergic neurons	No
	SBDS (5)	No	No
	HD (5,18)	No(5), Yes(18) / striatal neurons	No(5), Yes (18) / neurons contain CAG expansion
	SMA (11)	Yes / motor neurons	Yes / fewer motor neurons
	ASD (17)	Yes / functional neurons	Yes / neurons had fewer synapses, reduced spine density, smaller soma size, altered calcium signaling and electrophysiolosical defect
	PWS (75)	Yes / neurons	Yes / iPS cells showed reduced expression of disease associated small nucleolar RNA HBII85/ SNORD116
Immune system	ADA-SCID (5)	No	No
Endocrinology/Metabolism	GD (5)	No	No
	JDM (5)	No	No
	LNSc (5)	No	No
	Type1 diabetes (51)	Yes /pancreatic endocrine cells	Yes / insulin-producing, glucagon-responsive cells
Muscle skeletal	DMD (5)	No	No
	BMD (5)	No	No
Genetic	DS (5)	No	No
	LS (14)	Yes / cardiomyocytes	Yes / hypertrophic cardiomyocyte
	FD (10)	Yes / peripheral neurons	Yes / neural crest precurssors expressing low IKBKAP
	FRDA (9)	No	No / *FXN* gene repression and GAA• TCC repeat expansion are maintained in iPS cells
Hematological	MPD (7)	Yes / hematopoietic cells	No
	FA (8)	No	No
	β -thalassemia (13)	Yes / hematopoietic cells	No
Inherited liver disease	A1ATD (15)	Yes / hepatocytes	Yes / hepatocytes with polymeric A1AT
	GSD1a (15)	Yes / hepatocytes	Yes / hepatocytes with high intracellular glycogen amount
	GSD1b (16)	Yes / hepatocytes	No
	FH (16)	Yes / hepatoctyes	Yes / hepatocytes absent of LDL receptor
	CND (15, 16)	Yes / hepatocytes	No
	TYR1 (15, 16)	Yes / hepatocytes	No
	HER1 (16)	Yes / hepatocytes	No

figure 3 shows the different disease specific hiPSC lines created from patients. The disease abbreviations are explained below the figure. The figure also indicates if the cell lines were used for disease modeling, see last column (Chun, Chaudhari, & Jang, 2010).

ALS Amyotrophic lateral sclerosis PD Parkinson Disease SBDS Swachmann-Bodian-Diamond syndrome HD Huntington disease SMA spinal muscular atrophy ASD autism spectrum disorder PWS Prader Willi syndrome ADA-SCID adenosine deaminase deficiency related severe immunodeficiency GD Gaucher disease type III JDM juvenile diabetes mellitus LNSc Lesch-Nyhan syndrome DMD Duchenne muscular dystrophy BMD Becker muscular dystrophy DS down's syndrome LS LEOPARD syndrome FD familial dysautonomia FRDA Friedreichs ataxia MPD Myoloproliferative disease FA Fanconi anemia A1ATD Alpha 1 antitrypsin deficiency GSD1a Glycogen storage disease type 1a GSD1b Glycogen storage disease type 1b FH Familial hypercholesterolemia CND Crigler-Najjar syndrome TYR1 tyrosinemia type 1 HER1 progressive familial hereditary cholestasis MPD Myeloproliferative disorder.

Toxicity testing by hiPSC based cell assay.

According to the European Commission, there is almost no safety information for 99 percent of the tens of thousands of chemicals placed on the market before 1981 (Sinha, 2006). In 1981 when the last survey was held there were 100,106 chemicals in use in the EU (Sinha, 2006). Of these 100,106 chemicals only 3,000 have been tested for toxicity and over 800 of these 3000 chemicals are known to be carcinogenic, mutagenic or toxic to reproduction (Sinha, 2006). This is more than 25% of the chemicals tested which are carcinogenic mutagenic or toxic to reproduction. With the estimated cost of compliance being lower than the assumed health care benefits of saved billions of euros REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) was approved as legislation by the European Union. REACH is a European Union legislation which was proposed for two reasons: the protection of human health and the protection of the environment.

With almost no exceptions, toxicology testing in the 21st century relies on high dose testing in animals with some methods for extrapolating the results to levels of exposures in human populations. In addition, most of these toxicity tests focus heavily on explaining high dose rodent effects. However, it would be better to understand the biological basis for dose response relationships in humans exposed to chemicals at relevant environmental levels (Sinha, 2006). Also there is widespread disaffection with the use of high dose animal testing for toxicology from both regulatory agencies and the general public (Sinha, 2006). Using hiPSC the gap between animal model or immortalized cell lines and clinical trials can decrease (Inoue & Yamanaka, 2011).

For REACH about 100.000 chemicals have to be tested for toxicity. However, there are multiple forms of toxicity which all have to be tested for. These include amongst others; developmental toxicity, hepatotoxicity and cardiotoxicity which is split in electrophysical and biochemical toxicity (Anson et al., 2011). Hepatotoxicity, arises from a variety of mechanisms, including reactive metabolites, formation of reactive oxygen species, and inhibition of cytochrome P450 and/or transporter activity (Anson et al., 2011). A combination of tests is required to test a chemical's toxicity effect on all levels. To test toxicity with research animals only, the estimated number of research animals needed per chemical is a staggering 45 (Sinha, 2006). This is not a very strong number, however, it indicates the huge numbers of animals needed for REACH. This would mean that the total number of animals needed for REACH toxicity testing based on the number of chemical in 1981 becomes 4.5 million animals (Sinha, 2006). However, the use of current in vitro tests such as immortalized cell lines can reduce this number drastically to an estimated eight research animals per chemical which is already a huge reduction (Sinha, 2006). That figure works out to about 250,000 fewer animals used per year for toxicity testing alone and this reduction is without new improvements in hiPSC based cell assays which will have an even higher impact on the number of research animals needed per chemical (Sinha, 2006).

hiPSC based cell assays are expected to provide an innovative tool for toxicity testing for REACH. But what are the advantages of hiPSC based cell assays opposed to the current method of testing with immortalized cell lines and research animals? iPSC based cell assays can be generated from readily obtainable tissues, such as skin, peripheral blood, and other somatic tissues (Anson et al., 2011). This readily obtainable tissues can be used to generate large libraries of genetically diverse hiPSC based cell assays opening up multiple research options (Anson et al., 2011). In addition, the generation of hiPSC from persons who exhibit specific side effect profiles or idiosyncratic reactions to compounds may prove useful in screening for relatively rare but serious toxic effects (Anson et al., 2011). The most important advantage hiPSC have over immortalized cell lines is that although hiPSC have genetic artifacts from the Yamanaka transcription cocktail they are still genetically more intact then

normal immortalized cell lines making them react more normal to compounds (Anson et al., 2011). hiPSC haves advantages over research animals because research animals haves several drawbacks like the disaffection of the use in the population, species differences and lose in vivo phenotype and high variability from one isolation to the next (Anson et al., 2011).

Conclusion

hiPSC based cell assays are a promising alternative for the use of genetically altered cell based assays and research animals in disease modeling and toxicity testing. However, before hiPSC based cell assay can be fully used in disease modeling and toxicity testing substantial challenges remain to be overcome. The technologies used to generate hiPSC probably will continue its technological advance to help make the production of optimally reprogrammed hiPSC without genetic artifacts from the transduction possible and more efficient (Anson et al., 2011). The next big technological leap is to directly create the desired cell type by the use of tissue and lineage specific promoters so that the somatic cell can directly be reprogrammed into the cell of choice with high efficiency (Anson et al., 2011). Likewise, the first differentiation protocols directed toward other adult cell types such as spleen and kidney which are not yet made will broaden the landscape of potential toxicity assays (Anson et al., 2011). However, the relative maturity of the iPSC derived cell lineages remains a potential limitation, given that most cell types maintain a more embryonic phenotype in culture making the interpretation of the results harder (Anson et al., 2011).

However, disease modeling and toxicity testing by hiPSC based cell assay is a very promising tool in disease modeling and toxicity testing. There are only 2 major drawbacks of the use hiPSC based cell assays and these drawbacks are, it is not well possible to focus on different cell-cell interactions because it is hard to generate these interactions in vitro, and that it is not well possible to make a three dimensional cell based assay (Anson et al., 2011). However, it is possible to overcome this difficulty by studying different three dimensional cell-cell interactions in humanized systems such as immune compromised mice but then the problems with the use of research animals occur again (Inoue & Yamanaka, 2011).

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