Session 1: Stem cells and engineering

Lectures

L1.1

Induced pluripotent stem cells for research and therapy

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Induced pluripotent stem cells (iPSCs) are obtained by reprogramming of somatic cells, usually through overexpression of four transcription factors: Oct-4, Sox-2, Klf-4 and c-Myc. Development of these unique pluripotent cells able to differentiate into specialized cell types of all embryonic lineages was awarded with Nobel Prize to Shinya Yamanaka in 2012. Since 10 years of establishment of the first iPSCs numerous research and medical applications have been foreseen and tested. Reprogramming of patients' somatic cells to iPSCs generates stem cells with specific patient and disease characteristics. Therefore, besides the potential, although still quite distant use in regenerative medicine, the patient-specific iPSCs allow to investigate the disease mechanisms and drug testing in a way not offered previously by other techniques.

In this talk the strategies to generate murine and human iPSCs cells will be presented followed by demonstration of the methods of iPSCs differentiation into endothelial cells, cardiomyocytes, skeletal myoblasts and pancreatic-like cells. The role of heme oxygenase-1 in reprogramming will be discussed. The chances for the application of iPSCs technology for personalized precision medicine will be considered.

References:

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L1.2

Molecular signatures of Huntington disease pathogenesis are present in HD iPSCs and derived neurons

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Huntington disease (HD) is a brain disorder evoked by expanded CAG repeats in *HTT* gene which results in long polyglutamine tract in huntingtin protein (HTT). Interestingly, the subtle patient symptoms which are observed decades before overt HD symptoms and the molecular changes observed in cell and models demonstrate that the disease process begins earlier. The questions that remain are how early the molecular changes related to HD occur, and whether HD may be accounted as neurodevelopmental disorder. Therefore, it is essential to investigate the molecular signs of HD in cellular models reflecting the sequence of neurogenesis including pluripotent (e.g. iPS) and neural stem cells.

Therefore, we investigated naïve mouse HD YAC128 iPSCs and human HD iPSCs from HD and juvenile HD patients, to detect whether HD-related phenotypes can be already observed in pluripotent cells. Indeed, we found multiple alterations including the deregulation of the MAPK and Wnt signaling pathways and genes related to oxidative stress, such as SOD1. Interestingly, a common protein interactor of the huntingtin and the proteins in the above pathways is p53, and the expression of the p53 gene was deregulated in HD YAC128 iPSCs and human HD iPSCs. We further expanded the analysis to the iPS-derived neural stem cells (NSC) and found that p53 is also downregulated in HD NSC lines.

To investigate whether the expression of mutant huntingtin interferes with Wnt, Erk and p53 pathways we established isogenic HD-YAC128-iPSC and derived NSC lines with stable knockdown of mutant huntingtin expression by shRNA. We found that the level of mutant HTT expression modulated by shRNA is reversely correlated with the level of p53 resulting in normalization of its expression upon mutant HTT silencing. Investigation of shRNA expression lines delivered no clear evidence for changes in Wnt and Erk pathways upon huntingtin silencing in NSC and iPSC.

Our findings demonstrate that molecular HD signatures are already altered in undifferentiated pluripotent cells and that the pathogenesis of HD may start early in life. We also found that the deregulation continues along the CNS developmental cellular stages to the stage of Neuronal Stem Cells, illuminating HD as a putative neurodevelopmental disorder. An important molecule participating in early HD stages is p53, and its level in pluripotent and NS cells is dependent on the level of huntingtin therefore can be a valid molecular target for early therapy.

L1.3

Reprogramming of fibroblastic and epithelial cells with enhanced episomal system for generation of iPSC and their derivatives

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Induced pluripotent stem cells (iPSC) have the ability to proliferate indefinitely and the potential to give rise to virtually any cell type present in human body. Their prospective applications in the field of regenerative medicine provide novel, patient-personalized treatment options for numerous disorders and the replacement of diseased or damaged organ tissues.

In first part of this work, we focused on the ability of non-integrating oriP/EBNA-1 system to derive functional iPSC from somatic cells of various origin. We carried out a comparative analysis of iPSC generation efficiencies in fibroblasts and epithelial cells with transiently introduced episomal vectors. We determined that reprogramming efficiencies were significantly higher (up to 100 fold) in the epithelial cells compared with fibroblasts. Additionally, we demonstrated that the presence of microRNA cluster 302/367 in episomal vectors further enhanced reprogramming in both fibroblasts and epithelial cells, whereas the downregulation of Mbd3 expression improved iPSC colony-forming efficiency only in former cells.

The establishment of efficient methods to generate β-cell-like cells from human pluripotent cells currently remains a major challenge towards the development of cell-based treatment strategies for type 1 diabetes. For this purpose, we made an attempt to optimize differentiation protocols and created iPSC lines expressing PDX1 and NKX6.1 factors under the transcriptional control of tetracycline-regulated promoter. These cell lines were differentiated in xeno-free conditions using small chemical molecules in the sequential process through the definitive endoderm, pancreatic progenitors and insulin producing cells (IPC). Generated cells displayed molecular markers characteristic for respective steps of the differentiation. Notably, the obtained IPC released insulin and produced C-peptide with significantly higher secretion level in case of concurrent induction of PDX1 and NKX6.1 expression at the stage of maturation of progenitor cells.

Considering results obtained in the course of our work, we are strongly convinced that episomal reprogramming provides a simple, reproducible and efficient method for generating clinically relevant pluripotent cells. Furthermore, our data indicate that induced expression of PDX1 and NKX6.1 during certain steps of the differentiation process result in enhanced formation of cells sharing molecular features with cells originating from native pancreatic islets.

Oral presentations

O1.1

The influence of culture medium on ES cell differentiation

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Pluripotent stem cells (PSCs) can differentiate into all cell types building mammalian body. Their differentiation can be induced by the culture in suspension, in that spherical aggregates called embryoid bodies (EB) are formed. In EBs, PSCs differentiate into three germ layers: ecto-, endo-, and mesoderm, mimicking periimplantation stages of mammalian development. However, mesoderm and its derivatives are scarce in differentiating EBs. The differentiation of PSCs into mesoderm can be enhanced in the presence of factors inducing mesoderm formation in developing embryo. For example, it was reported that chemicals activating canonical Wnt signalling promote formation of mesoderm during *in vitro* differentiation of PSCs (Chal *et al.*, 2015, *Nat Biotechnol* **33**: 962-969.

Our study documents that the formation of germ layers can be affected by the composition of culture medium. We compared the morphology of EBs cultured in media containing either fetal calf serum of serum replacement. We also followed the expression of pluripotency and germ layer markers (mRNA and protein levels), proliferation of differentiating PSCs as well as their ability to adhere. We also compared the presence of markers of more specialized cells, such as neurons or hepatocytes, formed in the EB outgrowths cultured in such culture media. We found that cells cultured in the presence of fetal serum differentiate into endo- and mesodermal derivatives more efficiently than PSCs cultured under defined conditions, i.e. in medium supplemented with serum replacement. In the latter case we found more neuroectodermal cells.

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Posters

P1.1

Studying stem cells population dynamics and tracing cellular fate with iPLEX mass spectrometry

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The great promise that embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) hold for cell replacement therapies is hampered by their high heterogeneity and genomic instability. Cellular heterogeneity, studied mainly at molecular levels, is considered as an inherent property of most stem cell types, but acquired diversity in the population may arise during long term culture as well. Identification of major and minor genetic population changes appearing during culture is crucial for monitoring the genetic stability of pluripotent stem cells.

Genetic labelling technologies shed some light on the dynamics of stem and progenitor cell fate determination during development, organogenesis and tumorigenesis, however existing and applied methods used to identify the genetic labels are time-consuming, labour-intensive and require challenging analytical and computational steps for their identification.

Here, we report the novel strategy based on mass spectrometry, with greatly reduced complexity of analysis, for detection of DNA labels (barcodes) in stem cell population.

Designed genetic labels (DNA barcodes) were introduced into mouse embryonic stem cells by means of viral infections and were easily detected by mass spectrometry during routine cell culture. By mixing barcoded cell line showing growth advantage with normal barcoded population, we have studied the dynamics of cell culture repopulation.

Next, we have applied the developed method for studying the cell fate in early development of mouse embryo. We were able to detect single genetic labels in late blastocyst and in developing organs of foetuses, after introduction of barcodes and barcoded cells into 8-cell stage mouse embryo.

Advantages of presented method are various. Apart from short time-to-result and simple analysis, it is a versatile tool for studying population dynamics and controlled capture of single cell progeny thanks to the linkage of given barcode with targeted cell. The developed method can be easily applied for indirect monitoring of genetic stability in the population of iPSCs, as decrease in genetic diversity may reflect in gradual enrichment of mutant cells appearing in cell culture and concomitant accumulation of chromosomal aberrations.

P1.2

Nrf2 affects myoblast differentiation

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Nuclear factor (erythroid-related 2)-like 2 (Nrf2)is a transcription factor known as a master regulator of genes encoding oxidative stress- response and phase II detoxifying enzymes such as NQO1 and HO-1. Recently we have shown that overexpression of HO-1 inhibits myoblasts differentiation (Kozakowska *et al.*, 2012). Here we investigated the role of Nrf2 in that process.

C2C212 cells were stably transduced with retroviralvector harboring Nrf2, while control cells were transduced only with empty vetor. Differentiation was performed on confluent cells by changing medium to differentitation medium containing 2% horse serum instead of FBS. Cells were incubated in that condition for 5 days.

Nrf2 expressing cells showed enhanced proliferation and impaired differentiation as evidenced by decreased formation of myotube, accompanied by lower expression of MyoD and myogenin. Importantly, also the expression of myomiRs, includingmiR-206, miR-133a/b and miR-1 was downregulated in cells overexpressing Nrf2. Cells overexpressing Nrf2 demonstrated also increased expression of HO-1, as shown by RT-PCRand showed higher heme degrading activity, as evidenced by higher biliruibin production.Similarly, the expression of NQO1, another target of Nrf2, was upregulated. Moreover, in C2C12 overexpressing Nrf2 enhanced expression of p65 and YY1 was observed. In accordance with the antioxidant role of Nrf2, the generation of ROS was diminished in cells expressing Nrf2, both under basal and differentiation conditions.

Our data indicate that overexpression of Nrf2 affects myoblasts differentiation in a manner similar to observed in HO-1 ovexpressing cells. Induction of Nrf2 after muscle damage may thus be of significance for muscle regeneration. Prolonged activity of Nrf2 may, however, disturbproper myoblasts differentiation.

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P1.3

Lack of miR-378 improves dystrophic phenotype in *mdx* mice

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Duchenne muscular dystrophy (DMD) is a rare X-linked recessive genetic disorder caused by a mutation in gene encoding dystrophin. Inflammation is recognized as the key contributor to the pathogenesis of this disease, impairing the regenerative properties of satellite cells (SCs), the muscle progenitors. microRNAs, the small non-coding RNAs regulating the expression of majority of mRNA genes, have been recognized as the crucial regulators of proliferation and differentiation of SCs. Recently it has been demonstrated that miR-378 may play a role in myoblasts differentiation by inhibition of myoR, the repressor of MyoD (Gagan *et al*., 2011). Moreover miR-378 was shown to be upregulated in sera (Vignier *et al*., 2013) and downregulated in muscles (Roberts et al. 2012) of dystrophic mice. Therefore, we decided to elucidate the role of miR-378 in mice model of DMD (*mdx* mice).

We generated *mdx* mice lacking expression of miR-378 (miR-378$^{-/-}$*mdx*). To assess muscle functionality and performance, the animals were subjected to a downhill treadmill test and the running distance to exhaustion was measured. Interestingly, miR-378$^{-/-}$ mice ran twice the distance of WT animals. The double KO mice were stronger than *mdx* mice and run similar distance to control WT animals. LDH and CK activity was slightly increased in plasma of young miR378$^{-/-}$*mdx*, what could suggest higher muscle injury in these animals, however histological analysis did not confirm this observation. Decreased infiltration with inflammatory cells and decreased fibrosis in gastrocnemius muscles and diaphragms of young miR-378$^{-/-}$*mdx* was observed, what was fading with mice aging and progression of DMD. More detailed FACS data indicated similar number of SCs in miR-378$^{-/-}$*mdx* in comparison to *mdx* mice, however miR-378 appears to regulate their ability to differentiate and to form myotubes. Additionally increased ratio of M1/M2 macrophages was detected in miR-378$^{-/-}$*mdx*.

The results indicate for involvement of miR-378 in muscle damage. miR-378 exerts both satellite cells-specific effect and plays a role in regulation of inflammation in dystrophic muscles. Surprisingly, the lack of miR-378 might be beneficial in DMD.

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P1.4

From an idea to the pharma industry. Opportunities for bio laboratories

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Every project starts with an idea, but turning ideas into reality, especially in the pharma industry, is much more complicated. In the course of over 12-year experience in the R&D and regulatory field, SciencePharma has participated in challenging projects that were successfully brought to the commercialisation phase but also encountered development studies incorrectly designed and therefore of limited value from the regulatory perspective (e.g. due to not entirely clear requirements or even seemingly minor mistakes made at the initial stage). Full awareness of professional management, including regulatory support, is thus of key importance for successful development and commercialisation of new medicines, in particular complex biological products based on proteins, genes, cells or tissues. This is remarkably essential for small laboratories that sometimes either do not fully recognise constrains or do not discover hidden pearls in their projects.

This presentation aims to highlight some current opportunities for bio laboratories, both developing their own projects and carrying out studies on behalf of other enterprises.

Recently, of particular interest are advanced-therapy medicinal products (ATMPs), including gene therapy, cell therapy and tissue engineered products, as they offer entirely new prospects for the medicine. On the other hand they are highly challenging as they require compliance with various requirements, such as transplantation or GMP regulations as well as specific guidelines defining studies to be done before approval for putting on the market. Fortunately, alternative policies such as "hospital exemption" create possibility of early products commercialisation.

Another opportunity is developing "orphan" medicinal products, i.e. products intended for serious rare diseases. Such initiatives are significantly stimulated by the European Medicines Agency (EMA) through various incentives, e.g. market exclusivity or fee reductions.

Last but not least, bio laboratories may profit from recent approaches to substitute (whenever possible) studies in animals with in vitro assays. The latter are often significantly more sensitive than animal studies and thus may be highly useful in various cases, ranging from comparability studies for biosimilar products up to routine testing of many biological products.

Apart from above medium-sized, small and micro enterprises developing new products are supported by the EMA by regulatory assistance or fee reductions.

P1.5

Adipose derived stromal cells in heart regeneration

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Due to their therapeutic potential the adipose derived stromal cells (AdSC) may be used in regenerative medicine to treat post-infarcted heart. The most important advantage of the use of the cells isolated from adipose tissue is their accessibility, minimally invasive collecting procedure and possibility to obtain an abundant quantities. The aim of the study was to determine the conditions of AdSC isolation and *in vitro* culture and the feasibility of AdSC implantation into a murine model of myocardial infarction.

The material used in the study consists of human adipose tissues collected from anterior abdominal wall. Tissue was digested using collagenase NB4 and plated in DMEM medium containing 20% FBS and antibiotics. AdSC possessed all mesenchymal cells features: 1) were adherent to plastic (polystyrene) culture plates; 2) expressed surface antigens: CD105, CD73, CD29, CD44, CD90; and were devoid of hematopoietic (CD45, CD34), endothelial (CD31) cells antigens, and molecules of the major histocompatibility complex (HLA-DR); 3) differentiated into adipocytes, osteoblasts and chondroblasts. AdSC exhibit also a clonogenic potential. AdSC were injected directly into C57Bl/6NCrl mouse myocardium seven days after LAD ligation in amount of 500 000 cells per mouse. The control group consisted of mice injected with PBS^-. Two and six weeks after AdSC or PBS^- injections echocardiographic analysis were performed. Subsequently mouse hearts were removed for immunohistochemistry and immunofluorescence analysis in order to assess the progress of therapy.

Echocardiography showed an increase in left ventricular ejection in comparison with control group. Reduction in the size of the post-infarction scar and fibrosis as well as increase of vascularization in ischemic heart tissue was observed. Our results indicate that isolated human AdSC may be used to improve functionality of post-infarcted heart.

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P1.6

Therapeutic potential of cardiac mesenchymal stromal cells with $CD105^+CD34^-$ phenotype in a murine model of hindlimb ischemia

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It is well known that mesenchymal stromal cells (MSCs) participated in the repair of damaged tissues. It seems that MSCs mainly inhibit the inflammatory reaction and stimulate formation of blood vessels. The IL-6 secreted by MSCs plays a crucial role in this process. It is possible that IL-6 stimulates phenotypic changes from pro-inflammatory, antiangiogenic M1 macrophages to anti-inflammatory, proangiogenic M2 macrophages. The aim of the our study was to confirm proangiogenic properties of MSCs isolated from human hearts as well as participation of M2 macrophages in this process.

The MSCs with $CD105^+CD34^-$ phenotype were separated using a cell sorter. The cytokines and growth factors secreted by $CD105^+CD34^-$ cells were analyzed. The proangiogenic properties of MSCs were investigated in a murine model of hindlimb ischemia. The presence of IL-6 and M2 macrophages in ischemic tissue were determined by immunofluorescence staining. The effect of the conditioned medium from $CD105^+CD34^-$ cells on the phenotype of bone marrow-derived macrophages (BMDM) was also analyzed. The results suggest that IL-6 is the predominant cytokine secreted *in vitro* by the $CD105^+CD34^-$ cells. When $CD105^+CD34^-$ cells were administrated into ischemic muscle they retain their ability to secrete of IL-6. $CD105^+CD34^-$ cells injected into ischemic muscle an increase in the number of blood vessels in comparison to the control groups was observed. Also, around the implanted human cells an increase in the number of anti-inflammatory and proangiogenic M2 macrophages was observed. Moreover conditioned medium from $CD105^+CD34^-$ cells greatly increased the percentage of $CD206^+$ macrophages in BMDM culture.

Our results show that human cardiac mesenchymal stromal cells with $CD105^+CD34^-$ phenotype exhibit proangiogenic potentialin a murine model of hindlimb ischemia. The M2 macrophages are essential for healing and repair of damaged tissue.

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P1.7

Role of Nrf2 in acute and chronic muscle injury in Duchene muscular dystrophy

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Muscle injury is associated with the inflammatory response, which is necessary for proper amelioration of the damage, but when prolonged, impairs muscle regeneration. Inflammation in Duchenne muscular dystrophy (DMD) may result in upregulation of antioxidant genes like Nuclear factor (erythroid-related 2)-like 2 (Nrf2) and heme oxygenase (*Hmox-1*, HO-1). Nrf2 is a transcription factor regulating expression of several cytoprotective genes with antioxidant properties including *Hmox-1*. Recently we demonstrated the strong effect of HO-1 on myoblasts differentiation (Kozakowska et al, 2012). Therefore, here we aimed to investigate the role of Nrf2 in skeletal muscle injury and repair after cardiotoxin (CTX) injection and in mouse model of DMD (*mdx* mice).

To investigate the role of Nrf2 in muscle regeneration the WT and Nrf2 deficient mice (C57Bl/6) ($Nrf2^{-/-}$) were injected into gastrocnemius muscle with CTX. The treatment caused strong and fast induction of IL-6, IL-1b, HO-1, MCP1 and MIG in both WT and $Nrf2^{-/-}$ mice. In WT mice the expression of NQO1, a gene strictly regulated by Nrf2, was induced in skeletal muscle at day 1 and 3 after CTX injection, which was not the case in $Nrf2^{-/-}$ mice. Injury resulted in slightly induced muscle degeneration at day 1 in $Nrf2^{-/-}$ mice, with concomitant higher inflammation observed on day 3 after injection, as evidenced by increased LDH and CK plasma levels. Nevertheless, lack of Nrf2 does not affect final regeneration achieved at 28 days after injury, as evidenced by similar Myf5, MyoD, myogenin and myomiRs expression in both WT and Nrf2-deficient animals.

Interestingly, in *mdx* mice, under condition of chronic injury, decreased expression of NQO1 was detected in diaphragm, what stays in contrast to upregulation of NQO1 after CTX injury. Therefore to investigate the role of Nrf2 in progression of DMD, we generated *mdx* mice lacking expression of Nrf2 ($Nrf2^{-/-}$*mdx*). To assess muscle functionality and performance, $Nrf2^{-/-}$*mdx* mice were subjected to a treadmill test. $Nrf2^{-/-}$*mdx* mice had similar exercise capacity as *mdx* mice, indicating that lack of Nrf2 does not affect further muscle injury in dystrophic mice. This observation is confirmed by histological analysis showing similar level of muscle damage in $Nrf2^{-/-}$*mdx* and *mdx* mice.

In conclusion, the lack of Nrf2 results in moderate aggravation of muscle injury. However, lack of Nrf2 does not add to damage caused by dystrophin deficiency, indicating for the dispensability of Nrf2 in muscle regeneration.

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P1.8

The impact of Mesenchymal Stromal Cells (MSC) isolated from human adipose tissue on the hindlimb ischemia model in mice

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Mesenchymal stromal cells isolated from adipose tissue (ADSC ang. Adipose-Derived Stromal Cells) due to the availability of material, simplicity of isolation and the tremendous therapeutic potential raise a great hopes for the use in regenerative medicine. Mesenchymal stromal cells (MSCs) isolated from different human tissues may be transplanted into animals. MSC xenografts may be used in preliminary, preclinical studies. Nevertheless, the transplanted MSC are characterized by short retention times (most transplanted cells die within 48 hours after the transplantation). This short retention time should make MSCs more difficult to recognize by the recipient's immune system.

The aim of the project was to examine the impact of mesenchymal stromal cells isolated from human adipose tissue on the hindlimb ischemia model in mice.

The material used for the study was adipose tissue excised during surgeries in Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch (Poland). The study was conducted in accordance with the approval of the Local Committee on Bioethics in Katowice. To obtain the desired cell population the method of digestion of adipose tissue with collagenase solution was used. Phenotype of the cells was verified by flow cytometry. The hindlimb ischemia was induced by femoral artery ligation on males of the C57BL/6NCrl strain. After ligation, MSCs were administered into the femurs of studied mice. The control mice were injected with PBS. The function of the limb was assessed on days 1, 3, 7, and 14 following the artery ligation. The skeletal muscles of hindlimb were excised 14 days after ligation. Tissue sections were stained with H&E or with anti-CD31 antibody.

The established cell line has phenotype: $CD105^{+}/CD90^{+}/CD73^{+}/CD44^{+}/CD29^{+}/CD146^{+/-}$ and $CD45^{-}/CD34^{-}/HLA\text{-}DR^{-}/KDR^{-}/lin^{-}/CD31^{-}$. Isolated cells differentiate into adipocytes, chondroblasts and osteoblasts. We observed that ADSC-treated mice demonstrated an increase in capillary density comparing to controls. Furthermore we observed that ADSC injection into murine ischemic limbs improves functional outcomes.

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