Epigenetic regulation of cardiovascular differentiation

Kisho Ohtani and Stefanie Dimmeler*

Institute of Cardiovascular Regeneration, Center for Molecular Medicine, University of Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany

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Abstract

Epigenetic control mechanisms play a key role in the regulation of embryonic development and tissue homeostasis and modulate cardiovascular diseases. Increasing evidence suggests that lineage commitment of stem/progenitor cells is tightly regulated by epigenetic mechanisms. These epigenetic control mechanisms include DNA and histone modifications, which modulate the chromatin structure thereby regulating access of transcription factors. Particularly, the modification of histone acetylation and methylation, which is controlled by families of histone acetylases/deacetylases and methyltransferases/demethylases, respectively, controls stem cell maintenance, differentiation, and function. This review article summarizes our current understanding of epigenetic mechanisms regulating the differentiation of cardiovascular cells, specifically endothelial cells and cardiac muscle lineages. In particular, the article will focus on the enzymes which modify histones and are involved in chromatin remodelling.

Keywords

Epigenetics • Histone • Stem cells

This article is part of the Review Focus on: Epigenetics and the Histone Code in Vascular Biology

1. Introduction

Cell therapy is a promising option for the treatment of cardiovascular diseases. Although the treatment of patients with acute myocardial infarction with bone marrow-derived progenitor cells resulted in an overall improvement of heart function, and preliminary data suggested that the clinical outcome may also be affected,† the effects on ejection fraction were modest.‡ One reason for the limited effects might be the low capacity of the injected cells to differentiate into vascular cells and cardiomyocytes. Therefore, the understanding of the mechanisms regulating lineage commitment is important to programme adult cells to specific cardiovascular lineages.

Epigenetic control mechanisms play a key role in the regulation of tissue homeostasis and disease development. Mechanisms of epigenetic control include DNA and histone modifications, the regulation of mRNA stability and translation by non-coding RNAs (microRNAs) as well as differential RNA splicing. Despite advances in uncovering the molecular basis of these epigenetic mechanisms, their role in cardiovascular development, homeostasis and disease is largely unexplored. This review article summarizes our current understanding of epigenetic control mechanisms regulating the differentiation of cardiovascular cells, particularly endothelial cells and smooth and cardiac muscle lineages. A specific focus of this article is on histone-modifying enzymes involved in chromatin remodelling.

2. Epigenetic regulation by histone modifications

In the nuclei of eukaryotic cells, DNA is wrapped around an octamer of histone proteins that are packaged into high-order structures called chromatin. The chromatin structure is regulated by a variety of post-translational modifications including DNA methylation, modification of histones, and ATP-dependent chromatin remodelling. Histones can be modified by several post-translational mechanisms including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, or ribosylation of distinct amino acids, resulting in either activation or suppression of gene expression.§–‖ Enzymes that tightly control the balance of covalent histone modifications are histone acetyltransferases (HATs) and deacetylases (HDACs) as well as histone methyltransferases (HMTs) and demethylases (HDM) (Figure 1). These enzymes alter the configuration of the chromatin and regulate gene expression. Acetylation of lysine residues in histone tails by HATs unpacks chromatin structure and renders the DNA accessible to transcription factors. The effects of HATs are counteracted by HDACs, which pack chromatin and repress gene transcription. In mammals there are 18 HDACs, which are divided into four distinct classes based on sequence homology to yeast HDACs and functional similarities. Class I HDACs

* Corresponding author. Tel: +49 69 6301 6667, fax: +49 69 6301 6374, Email: dimmeler@em.uni-frankfurt.de

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(1, 2, 3, and 8) are primarily located in the nucleus and are ubiquitously expressed. Class II HDACs are divided into two subgroups: IIa HDACs (4, 5, 7, and 9) and IIb HDACs (6 and 10), which shuttle between the nucleus and cytoplasm and show a tissue-specific expression pattern.9,10 The NAD$^+$-dependent enzymes of Class III HDACs (also named SIRTUINS) comprise seven members (SirT1–7) and are ubiquitously expressed. Although most of these enzymes were shown to regulate histone acetylation, their distinct biological functions are largely due to the deacetylation of non-histone proteins such as transcription factors.

In contrast to histone acetylation, histone methylation is rather complex, and the position of the targeted amino acid as well as the degree of methylation (mono-, di-, or trimethylation) determine the overall structure of chromatin and the effect on transcription (Figure 1). Methylation occurs either at lysine residues at different positions, e.g. at lysine 4 or lysine 27 (abbreviated as K4 or K27, respectively) or at arginine residues. For example, an open chromatin, which is permissive for gene activation, is associated with the accumulation of trimethylation of lysine 4 of histone H3 (abbreviated as H3K4) (Figure 1). On the other hand, a closed chromatin is associated with trimethylation of H3K9, H3K27, or trimethylation of K20 of histone 4. Recent genome-wide studies of histone modifications confirmed that histone modifications control the chromatin state and gene transcription.11–14 Histone methylation was thought to be a stable and irreversible epigenetic mark, but recently several groups showed that histone demethylases can remove methyl groups, making the regulation of histone methylation more dynamic.15 These complex histone modifications add a new layer of regulation for the control of gene expression, which are essential for appropriate cell fate decision and organogenesis. Therefore, understanding of epigenetic regulation will be essential to determine what guides cells to specific lineages and thus may also provide therapeutic options for cell therapy of cardiovascular diseases.

2.1 Histone-modifying enzymes and vascular differentiation

2.1.1 Histone acetylation and vascular differentiation

The first evidence that histone acetylation plays a role in endothelial differentiation comes from studies using pharmacological broad-spectrum HDAC inhibitors such as trichostatin A (TSA). These inhibitors were shown to reduce the expression of endothelial marker proteins in adult bone marrow- or circulating blood-derived endothelial progenitor cells.16 These data are consistent with previous findings showing that angiogenic functions and the expression of endothelial nitric oxide synthase (eNOS) were reduced by TSA or valproic acid, another HDAC inhibitor.17,18 However, subsequent studies closely evaluated the effect of HDAC inhibitors, and it was shown that, when using lower concentrations of the inhibitors, eNOS expression was augmented, indicating a striking dose-dependent effect of HDAC inhibitors.19 Apart from the critical influence of the concentration of HDAC inhibitors on eNOS expression, the effect may also depend on the cell type treated. Thus, Fish et al.20 reported that TSA treatment resulted in an up-regulation of eNOS expression in non-endothelial cells such as smooth muscle or HELA cells, whereas TSA reduced eNOS under the same experimental conditions in endothelial cells. These results may indicate that under conditions of pronounced histone acetylation of the eNOS promoter—as it

![Figure 1]: Schematic illustration of the regulation of chromatin structure. The assembly of epigenetic modulating enzymes controls chromatin structure. Heterochromatin prevents the access of transcription factors (TF), whereas euchromatin is accessible for transcriptional activation. These structures are controlled by histone modifications and chromatin remodelling factors. K, Lysine; Me, Methyl group; Ace, Acetylation; PRC, Polycomb repressor complex.
occurs in endothelial committed cells—TSA has no effect on the histone acetylation state of the eNOS promoter and may induce proteins that secondarily negatively control eNOS expression. In contrast, in non-endothelial cells, which are characterized by a low histone acetylation mark at the eNOS promoter, TSA increases the histone acetylation and can derepress the eNOS promoter activity. Indeed, in bone marrow-derived multipotent adult progenitor cells histone deacetylase inhibitors, in combination with inhibition of DNA methylation by 5′-aza-2′-deoxycytidine, induced differentiation into endothelial cells. DNA methyltransferase inhibition alone also induced endothelial cell differentiation in mouse embryonic stem cells (ESC). Interestingly, NO, which is generated by eNOS, has a feed-back effect via activating histone deacetylases and thereby controlling mesoderm differentiation of mouse ESC.

Of note, the pharmacological HDAC inhibitors used in the cited studies are not specific for one HDAC isoform. Since it is well-known that the individual HDACs can have specific and even antagonizing effects, it is conceivable that the biological effects seen after global inhibition of HDACs may depend on the expression pattern of specific HDACs that are biologically relevant and active in a given cell type. With respect to endothelial differentiation, particularly the isoform HDAC3 gained increasing attention. HDAC3 is crucial in ESC differentiation toward endothelial cells and plays an important role in mediating flow responses. Laminar flow is well-known to augment endothelial differentiation of peripheral blood- and bone marrow-derived progenitor cells and ESC. and it induces and/or activates HDAC1, HDAC3, and HDAC5. Silencing of HDAC3 specifically blocked basal and flow-stimulated expression of endothelial marker genes in ESC, suggesting that HDACs are crucial regulators of endothelial differentiation. However, in contrast, shear stress also was shown to stimulate histone acetyltransferase activity, which was associated with increased eNOS expression in endothelial cells and endothelial marker gene expression of mouse ESC. How these contrasting data showing that activation of HDACs and HATs are both involved in flow responses and control endothelial gene expression can be explained remains to be determined.

In genetic studies, HDAC7-deficient mice showed the most impressive vascular phenotype, including a failure in endothelial cell–cell adhesion and consequent dilatation and rupture of blood vessels. However, this severe defect was not related to an effect on endothelial differentiation. HDAC5 also regulates endothelial cell functions; however, in contrast to HDAC7, it appears to act as a negative regulator.

Among the class III NAD+-dependent HDACs, SIRT1 was shown to be essential for postnatal neovascularization. However, the essential role of SIRT1 is rather due to effects on the molecular signalling pathways that regulate the function of mature endothelial cells instead of influences on the differentiation of progenitor cells towards the endothelial lineage. Despite the lack of evidence for a direct role of SIRT1 in endothelial differentiation, pharmacological activation of SIRT1 by resveratrol, a component of red wine, improved endothelial progenitor cell numbers and functions in several studies.

**2.1.2 Histone methylation and vascular differentiation**

Histone methylation critically regulates gene expression. With respect to endothelial cell differentiation, earlier studies suggested the involvement of the HMT Ezh2, which is part of the polycomb repressor complex 2 (PRC2). PRC2 comprises polycomb group (PcG) proteins, which control the expression of genes essential for cell fate and embryogenesis and repress targeted genes by methylation of H3K27 (Figure 1). In detail, silencing of Ezh2 and other components of the PRC2 induced the re-expression of the Ephrin receptor B2 and other endothelial genes in A673 tumour cells. Furthermore, endothelial tube formation of several different tumour cell types was shown after silencing of Ezh2, suggesting that Ezh2 maintains a stemness signature and is a negative regulator of endothelial commitment and angiogenesis. These findings, however, differ from those of others in which VEGF was shown to augment Ezh2 expression in endothelial cells, leading to the silencing of the angiogenesis inhibitor vasohibin 1 and thereby stimulating tumour angiogenesis. The conflicting results of the two studies might be related to the different effect of Ezh2 in tumour cells vs. endothelial cells: in tumour cells Ezh2 maintains stemness and thereby blocks endothelial differentiation whereas in mature endothelial cells, Ezh2 blocks angiogenesis.

The lysine methyltransferase (MLL), which mediates H3K4 methylation and thereby activates gene expression, was shown to regulate endothelial cell functions (Figure 1). Silencing of MLL prevented the expression of homeobox transcription factors such as HoxA9 and HoxD3, which are both important for the transcriptional control of several endothelial genes such as eNOS and EphB4. The role of MLL in endothelial differentiation has not been explored, but likely its influence on transcription factors known to control endothelial commitment also affects endothelial differentiation.

Another methyltransferase that was shown to control vascular differentiation is the histone H3K36 methyltransferase Hypb/Setd2. Hypb−/− mice die during embryonic development at embryonic day 10.5 (E10.5) to embryonic day 11.5 (E11.5) and show vascular remodelling defects. Further insights were gained by inducing differentiation of Hypb−/− ESC. Although some CD31-positive cells were obtained, vessel formation, migration and tube formation was significantly impaired, indicating that the methyltransferase Hypb is required for proper vessel development in vivo and in vitro.

### 2.2 Histone-modifying enzymes and cardiac differentiation

Cardiac lineage differentiation and development is a well-organized process that requires the intimate interaction of transcription factors such as the homeodomain protein Nkx2.5, the MADS box factor MEF2c, the zinc finger domain protein GATA-4, and the T-box transcription factor Tbx5. Chromatin-modifying factors are closely associated with these transcriptional networks, and the epigenetic control of these cardiac transcription factors as well as their targets by chromatin-modifying enzymes is involved in proper cardiac differentiation and development.

#### 2.2.1 Pharmacological histone modification and cardiac differentiation

To enhance cardiac differentiation several pharmacological compounds have been examined. 5-Azacytidine, an inhibitor of DNA methylation, promotes cardiac differentiation in a time-dependent manner in embryonic stem cells and adult mesenchymal stem cells. In addition, inhibition of HDAC by TSA also promotes cardiac differentiation by increased expression of acetylated GATA4, NKx2.5, and MEF2c. Although the enhancement of cardiac differentiation by pharmacological modifiers might be attractive for increasing the yield of cells for cell therapy, such strategies
warrant further meticulous investigation because TSA (and likely other epigenetic regulators) has distinct effects depending on the type of cell and the stage of differentiation.\textsuperscript{51–53}

### 2.2.2 HDACs and cardiac differentiation

HDACs initially gained attention as crucial regulators of cardiac hypertrophy. Genetic knockout studies, however, have also revealed that HDACs play a fundamental role in cardiac differentiation and development (Table 1). Thus, specific but partially overlapping functions have been assigned to individual HDAC isoforms.\textsuperscript{54,55} As summarized in Table 1, mice with cardiac-specific knockout of the individual HDAC isoforms HDAC1 or HDAC2 and mice with constitutive knockout of HDAC5 and HDAC9 are viable and did not exhibit cardiac developmental defects. However, the combined deletion of HDAC1/2 and HDAC5/9 resulted in more severe defects, suggesting that HDAC isoforms may have partially overlapping and redundant functions.

The mechanisms underlying the control of cardiac differentiation by HDACs are not entirely clear, but, based on the finding that class II HDACs directly bind and repress MEF2 in adult cardiomyocytes,\textsuperscript{56,57} cardiac developmental defect in class II HDAC mutant mice might be attributable to the derangement of the cardiac transcription factors network.

In contrast, inhibition of class III HDAC SIRT1, which is highly expressed in the murine embryonic heart, leads to perinatal or postnatal lethality, and the mice displayed cardiac developmental defects, including septal defects.\textsuperscript{58,59} SIRT3- or SIRT7-deficient mice are viable, suggesting that these two members of the SIRT family are not essential for embryonic cardiac development. However, these knockout mice develop cardiac hypertrophy after birth.\textsuperscript{60,61}

Together, genetic studies demonstrate that HDACs regulate cardiac development and hypertrophy, although the function of some isoforms might be partially compensated by other family members. The direct involvement of HDACs in cardiac stem cell function is less clear and deserves further study.

### 2.2.3 HATs and cardiac differentiation

Several HATs are known to regulate cardiac gene expression.\textsuperscript{62–64} Among them, the E1A-associated nuclear protein p300 is most intensively studied in cardiac development. Homozygous p300 knockout mice die between 9 and 11.5 days of gestation, exhibiting defective heart development with reduced trabeculation and reduced expression of cardiac structural proteins such as α-MHC and α-actinin.\textsuperscript{65,66} A genetic knock-in study showed that the acetyltransferase activity of p300 is essential for cardiac organogenesis.\textsuperscript{67} However, cardiac-specific p300 expression mice show cardiac

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The phenotype of homozygote knockout mice is described.

Table 1: Cardiac phenotype of mice lacking epigenetic enzymes

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hypertrophy and heart failure with increased mortality,\textsuperscript{68–70} demonstrating that the level of p300 needs to be tightly controlled. In addition to the role as acetyltransferase of histone tails, p300 also acetylates non-histone proteins\textsuperscript{71} and interacts with cardiac transcription factors such as GATA4, myocyte enhancer factor-2 (MEF-2), and serum response factor to increase their DNA binding.\textsuperscript{49,72–76}

\subsection*{2.2.4 HMTs and cardiac differentiation}

Despite increasing evidence suggesting the participation of histone methylation in the regulation of cardiac development,\textsuperscript{11,12} the specific role of histone methylation in the processes regulating cardiac differentiation are less well-known compared with the established roles of HATs and HDACs. This is partly due to the fact that most mice lacking the HMTs Eed, Ezh2, Suz12, Suv39h, and G9a die at very early post-implantation stages,\textsuperscript{77–81} which precludes further analysis. Additionally, studying histone methylation is more complex since the different acceptor amino acids and extent of methylation (e.g. mono-, di-, or trimethylation) can have different effects. However, the function of some HMTs in cardiac development and differentiation has been elucidated in genetic models. The Wolf–Hirshhorn syndrome candidate 1 (WHSC1) gene is a H3K36 trimethyltransferase that is essential for cardiac development. Histone trimethylation at lysine 36 (H3K36me3) is associated with active gene transcription.\textsuperscript{11} Homozygous deletion of the WHSC1 gene leads to atrial and ventricular septal defects and the mice die soon after birth.\textsuperscript{82} These phenotypes resemble the Wolf–Hirshhorn syndrome, and WHSC1 interacts and collaborates with Nkx2.5 to repress the transcription of Nkx2.5 target genes in embryonic heart, presumably mediated by H3K36me3.\textsuperscript{83} Another methyltransferase that has been linked to cardiac development is Dot1L, which catalyses H3K79 methylation despite lacking a SET domain.\textsuperscript{84} Dot1L knockout mice are embryonically lethal, exhibiting a severe phenotype including dilated heart and angiogenesis defects in the yolk sac, and H3K79 methylation is globally lost in Dot1L-deficient ESC displaying aberrant telomere elongation and proliferation defects.\textsuperscript{85}

Furthermore, knockout of Rae28, a member of the polycomb repressive complex 1 that catalyses mono-ubiquitylation of histone H2A and regulates gene silencing, leads to cardiac abnormalities compatible with tetralogy of Fallot (TOF) and double-outlet of the right ventricle (DORV). Rae28 helps to maintain the expression of Nkx2.5.\textsuperscript{86} Smyd1, a member of the SET and MYND domain-containing (Smyd) family that has methyltransferase activity, is also essential for cardiac development. Smyd1-deficient mice are embryonically lethal due to disrupted cardiomyocyte maturation and a right ventricular developmental defect. Smyd1 controls the expression of Hand2 and serves as a downstream effector gene of MEF2c.\textsuperscript{88–90} The precise mechanisms underlying transcriptional control by Smyd1 remains to be deciphered, and in vitro studies suggest Smyd1 functions via an interaction with other chromatin remodelling factors.\textsuperscript{88,91,92}

\subsection*{2.2.5 HDMs in cardiac differentiation}

The methylation of histones has been considered to be a stable (irreversible) epigenetic modification. However, the recent discovery of histone demethylases, including the amine oxidase LSD1 and Jumonji C (JmjC) domain proteins, has changed this view and suggested that histone methylation might be reversible, allowing a dynamic regulation\textsuperscript{5,15} (Figure 1). Whereas LSD1 can only catalyse demethylation of mono- and dimethylated lysines, JmjC-domain containing demethylases were shown to demethylate mono-, di-, and trimethylated lysines. There is increasing evidence that histone methylation contributes to the maintenance of embryonic stem cells and proper differentiation and development\textsuperscript{13,14,93} (Figure 2).

One of the first members of the family of JmjC domain proteins that has been linked to cardiac development is Jarid2, also known as Jumonji.\textsuperscript{94,95} Jarid2 is expressed in the developing and adult
heart,96 and depending on the genetic background of the mouse strains, Jarid2 knockout mice die embryonically97 or perinatally96 and exhibit cardiac malformations. Cardiomyocytes in Jarid2 null mice were differentiated, but the transcriptional regulation of genes involved in heart chamber formation was disturbed in late-stage embryos.96,98 The molecular mechanisms underlying the transcriptional repression caused by Jarid2 is rather complicated because, unlike other Jmjd domain-containing proteins, Jarid2 is devoid of histone demethylase activity.99 However, Jarid2 was shown to repress the expression of atrial natriuretic factor and α-MHC through interaction with Nlx2.5, GATA4, retinoblastoma protein, and MEF2c.98,100–103 The repressive activity of Jarid2 is considered to be mediated by recruitment of H3K9 methyltransferase G9a and the GLP complex at the promoter of cyclin D1104 and by recruitment of PRC2 to target genes.105–108

Another Jmjd domain-containing protein, Jmjd6, is also essential for development, and Jmjd6-deficient mice show a delay in terminal differentiation of multiple organs109 and perinatal lethality due to cardiac development, and Jmjd6 mutant mice show a delay in terminal differentiation of beating cardiomyocytes from non-cardiogenic mesoderm.119

transcription factors (GATA4, Nkx2.5, and Tbx5) resulted in induc- tional repression caused by Jarid2 is rather complicated because, unlike other JmjC domain-containing proteins, Jarid2 is devoid of histone demethylase activity.113,114 BAF complexes are evolutionarily conserved large DNA–nucleosome interactions (Figure 1).13,14 BAF complexes are essential to maintain the pluripotency of embryonic stem cells.115,116 Interestingly, these complexes change the subunit isoforms and their tissue-specific functions during differentiation from embryonic stem cells to cardiac lineage cells. Baf60c, a subunit of the BAF complexes, is expressed in the developing heart and is required for cardiac morphogenesis and establishment of left–right asymmetry.117,118 The addition of Baf60c to the cocktail of cardiac transcription factors (Gata4, Nlx2.5, and Tbx5) resulted in induction of beating cardiomyocytes from non-cardiogenic mesoderm.119

Baf60c reinforces the function of cardiac transcription factors such as Gata4 and Tbx5 by acting as a bridge between transcription factors and their target genes. Brg1 is another ATPase subunit of the BAF complex that also plays a fundamental role in the developing and adult heart. Cardiac-specific Brg1 knockout mice are embryonically lethal due to loss of a compact myocardium and an absent septum.120 Mice with endocardium-specific Brg1 deletion exhibit trabeculation defects resulting from derepression of a secreted matrix metalloproteinase, ADAMTS1, which prevents excessive trabeculation.121 Brg1 also regulates the isoform shift from α-myosin heavy chain to β-myosin heavy chain in embryonic heart and in pathological hypertrophy via interaction with HDACs and poly(ADP-ribose) polymerase (PARP).122 These studies indicate that ATP-dependent chromatin remodelling is involved in cardiac differentiation.

3. Summary and conclusions

Although increasing evidence suggests that histone-modifying enzymes play crucial roles in the cardiovascular system, their specific function in cardiovascular lineage commitment and differentiation is not entirely clear. Often, conflicting data are obtained in studies pharmacologically interfering with histone modifications. The reason for these conflicting reports may in part be the different responses of cells at different states of differentiation. As illustrated in Figure 2, differentiation requires both the repression of the pluripotency genes and simultaneously the up-regulation of lineage markers. For allowing appropriate differentiation to one lineage, other lineages additionally need to be suppressed. All of these processes are regulated by epigenetic control mechanisms and partially by the same enzymes. Therefore, proper differentiation requires promoter-specific regulation of chromatin structures. Further studies are necessary to fully understand the recruitment of enzymes that regulate chromatin remodelling and histone modifications.

A second challenge in studying histone-modifying enzymes relates to their multiple functions. As discussed in this article, most if not all histone-modifying enzymes also regulate other targets and often directly control transcription factor activities and signalling pathways as well. Therefore, before targeting epigenetic enzymes for modulating cell fate decisions, the molecular mechanisms need to be more precisely identified.

Despite these open questions and concerns, the transient manipulation of enzymes involved in epigenetic control may be useful to facilitate reprogramming of cells for cell therapy. The finding that overexpression of the chromatin remodelling enzyme Baf60c potenti- ates the function of the transcription factors Gata4, Nlx2.5, and Tbx5 to induce cardiac differentiation may be a first example of how the modulation of chromatin remodelling may be useful to drive cardio- genesis. However, thus far only embryonic or extraembryonic tissue has been used in these experiments. In adult cells, the combination of the three transcription factors Gata4, Mef2c, and Tbx5 alone was recently shown to directly reprogramme fibroblasts into functional cardiac myocytes,122 and it remains to be determined whether the addition of epigenetic modifiers indeed facilitates reprogramming of adult cells.

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References

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