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REVIEW

Modeling murine yolk sac hematopoiesis with embryonic stem cell culture systems

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Abstract **The onset of hematopoiesis in mammals is defined by generation of primitive erythrocytes and macrophage progenitors in embryonic yolk sac. Laboratories have met the challenge of transient and swiftly changing specification events from ventral mesoderm through multipotent progenitors and maturing lineage-restricted hematopoietic subtypes, by developing powerful *in vitro* experimental models to interrogate hematopoietic ontogeny. Most importantly, studies of differentiating embryonic stem cell derivatives in embryoid body and stromal coculture systems have identified crucial roles for transcription factor networks (e.g. *Gata1*, *Runx1*, *Scl*) and signaling pathways (e.g. BMP, VEGF, WNT) in controlling stem and progenitor cell output. These and other relevant pathways have pleiotropic biological effects, and are often associated with early embryonic lethality in knockout mice. Further refinement in subsequent studies has allowed conditional expression of key regulatory genes, and isolation of progenitors via cell surface markers (e.g. FLK1) and reporter-tagged constructs, with the purpose of measuring their primitive and definitive hematopoietic potential. These observations continue to inform attempts to direct the differentiation, and augment the expansion, of progenitors in human cell culture systems that may prove useful in cell replacement therapies for hematopoietic deficiencies. The purpose of this review is to survey the extant literature on the use of differentiating murine embryonic stem cells in culture to model the developmental process of yolk sac hematopoiesis.**

Keywords hematopoietic, progenitors, embryonic, stem cells, differentiation

An introduction to embryonic hematopoiesis in the mouse

Hematopoiesis begins early in mouse embryonic development, when blood islands are specified in the extra-embryonic mesodermal tissue of the yolk sac (YS). Blood islands contain both hematopoietic and endothelial precursors, and mark the site of the first wave of hematopoiesis around day E7.5 (Palis et al., 1999), which generates nucleated, comparatively large erythrocytes that express embryonic globins (Fig. 1A). The YS program additionally generates limited progenitors of the myeloid and megakaryocyte lineage. The YS events are typically referred to as the primitive wave of hematopoiesis, and are distinguished from subsequent embryonic and postnatal events, which comprise definitive hematopoiesis. The para-aortic splanchnopleura (P-Sp) and aorta-gonado-mesonephros (AGM) regions serve as intermediate sites of hematopoiesis to generate the first definitive progenitors including long-term hematopoietic stem cells. With the onset of circulation, progenitors seed the fetal liver (Lux et al., 2008), the next and final site of hematopoiesis before birth (Fig. 1A). During this transition, the transient wave of primitive erythrocytes (EryP-CFC) dissipates by about day E9.0 (Palis et al., 1999), although vestigial EryP-CFC populations enucleate and persist into neonatal circulation as primitive erythrocytes (Kingsley et al., 2004). In parallel, smaller, definitive erythrocytes (EryD), which lack nuclei and express exclusively adult globins, emerge from the fetal liver; these eventually mature to become functional circulating red blood cells. The stem and progenitor cells responsible for generating mature hematopoietic sub-types of all lineages finally reside in the bone marrow, which serves as the site of hematopoietic renewal throughout life (Fig. 1A).

Embryonic hematopoiesis is therefore a highly complex process composed of distinct developmental programs and is

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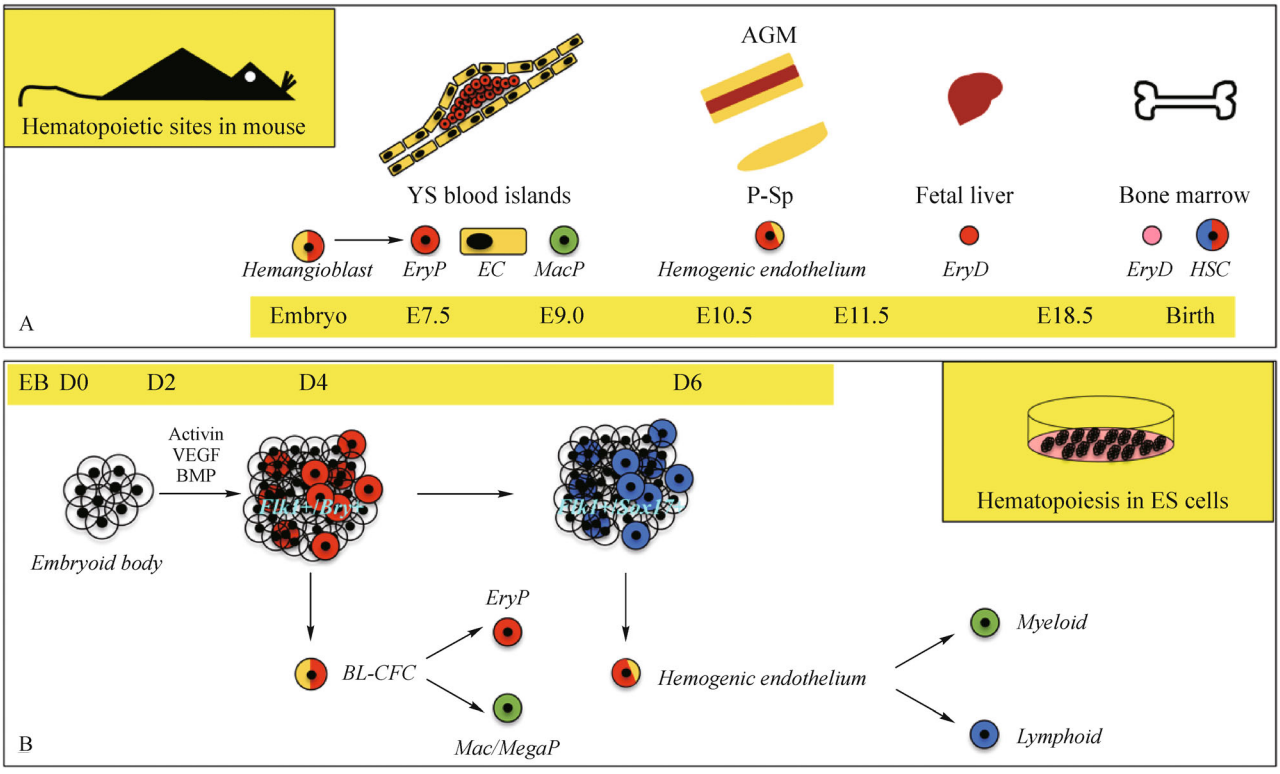

Figure 1 Differentiating embryonic stem cells as a model system for embryonic hematopoiesis. (A) A schematic of hematopoietic transitions in the developing mouse embryo, showing the approximate embryonic developmental time frame associated with each site. Also shown are key cell types that arise at each site/time point, with the first events generating primitive erythroid progenitors and endothelium in the extra-embryonic yolk sac (YS) from ventral mesoderm-derived hemangioblast. Hemogenic endothelium generated in P-Sp and AGM begins the definitive wave that marks the transition to the fetal liver and finally the bone marrow as the site of hematopoietic renewal via adult HSCs. Abbreviations: Para-aortic splanchnopleura (P-Sp); aorta-gonad-mesonephros (AGM); endothelial cell (EC); primitive and definitive erythrocyte (EryP and EryD); hematopoietic stem cell (HSC). (B) Schematic of analogous hematopoietic progenitor development in murine ES/EB cultures, with timeline highlighted in yellow and mirrored to embryonic development. Specification of progenitors proceeds from mesoderm generated in early EBs stimulated to adopt hemato-vascular fate by Activin, VEGF, and BMP, resulting in a BL-CFC population that is $FLK1^{+}/BRY^{+}$ and equivalent to the hemangioblast, capable of generating hematopoietic colonies equivalent to progenitors from yolk sac. A second $FLK1^{+}$ cell population co-expresses SOX17, and functions similarly to hemogenic endothelium, with potential for definitive hematopoietic lineages including lymphoid cells.

subject to many potential deleterious perturbations. Although, for instance, primitive erythroid progenitors are transient in nature, they mark the essential first step in establishing the embryonic hematopoietic system. Impairment of these early hematopoietic events is incompatible with further development in mouse models. Studies have thus focused on embryonic hematopoietic development from several different perspectives, including but not limited to the following types of investigations: 1) Understanding the processes of specification of mesodermal precursor populations fated to differentiate into hematopoietic progenitors; 2) Elucidating the signaling pathways and gene expression patterns responsible for differentiation and expansion of these progenitors; and 3) Identifying conditions in which hematopoietic progenitor output can be induced to expand or contract, accelerate or delay, with the ultimate goal of directing production of progenitor cells that are potentially clinically relevant in replacement therapies for disorders marked by hematopoietic failure. From these three strategies, two important broad observations have emerged. First, manipulation of embryonic stem (ES) cell cultures is a particularly useful experimental platform to study hematopoietic ontogeny. Secondly, several key genetic programs and signaling pathways exert regulatory control in a context-dependent manner throughout development. For the purpose of brevity, this review will focus on studies of murine yolk sac hematopoiesis, but will additionally reference how they have influenced ongoing attempts to isolate therapeutic cell types through directed differentiation.

Different paradigms of embryonic stem cell differentiation: powerful tools to model yolk sac hematopoiesis *in vitro*

Efforts to characterize early murine hematopoietic develop-

ment identified YS blood islands as the sites of initiation. However, the relative inaccessibility of developmentally relevant progenitors with rapidly shifting potentials prompted a shift in strategy to model the process *in vitro* using the mouse ES cell system. Embryonic stem cells can be isolated from the inner cell mass of the mouse blastocyst. They are both pluripotent and self-renewing, and can differentiate into precursors from all three primary germ layers and many subsequent derivatives. The classic system for measuring differentiation potentials within ES cell-derived populations is the embryoid body (EB). EBs typically develop as heterogeneous clusters of cells in suspension after removal of leukemia inhibitory factor (LIF), a cytokine that maintains pluripotency. Early studies found that EBs faithfully recapitulated the development of YS blood island-like cell types, suggesting that EB cultures intrinsically contain hematopoietic potential (Doetschman et al., 1985). Protocols to generate erythroid colonies *in vitro* from murine hematopoietic tissue sources (Stephenson et al., 1971; McLeod et al., 1974) influenced later efforts to quantify and manipulate hematopoietic output in other cell culture systems. Hematopoietic output in EBs can be augmented in a lineage-restricted manner via addition of specific cytokines, such as erythropoietin (EPO) for EryP (Wiles and Keller, 1991), and interleukin-3 (IL-3) and macrophage colony-stimulating factor (M-CSF) for macrophage progenitors (Wu et al., 1995; Lichanska et al., 1999). Further analysis noted that hematopoietic development in EBs closely mirrors the stepwise progression of YS hematopoiesis, proceeding from precursors enriched for mesodermal marker transcripts to primitive erythroid, macrophage, definitive erythroid, and multilineage progenitors (Keller et al., 1993).

A seminal event in the evolution of ES/EB model systems as surrogates for developmental hematopoiesis was the identification by Keller and colleagues of the bi-potential hemato-vascular progenitor equivalent to the putative hemangioblast. They found that stimulation of EB cultures on day 3–3.5 of differentiation with vascular endothelial growth factor (VEGF) and kit-ligand (SCF) promoted expansion of blast colony-forming cells (BL-CFC), a progenitor cell type with primitive and definitive hematopoietic, as well as endothelial, potential (Kennedy et al., 1997; Perlingeiro et al., 2003) (Fig. 1B). The BL-CFC is sensitive to ectopic expression of the homeobox gene *Hox11* (Keller et al., 1998), manipulation of LIF/STAT (signal transducer and activator of transcription) signaling (Chan et al., 2003; Zou et al., 2006) and genetic deletion of runt-related transcription factor 1 (*Runx1*) (Lacaud et al., 2002). Further analysis in human ES/EB culture systems identified analogous progenitors with selective potentials for erythroid versus myeloid output (Kennedy et al., 2007). These studies and others in mouse and human systems (Perlingeiro et al., 2001; Wang et al., 2004; Zambidis et al., 2005; Tober et al., 2007; Gandillet et al., 2009) revealed the potential for stem/progenitor populations that were profoundly useful experimentally. Specifically, these cell types represented a discrete demarcation between multipotency and fate restriction, and prompted a cogent argument for the importance of instructive signaling within a hematopoietic niche.

Alternative cell culture platforms using adherent monolayers have also been developed for the study of hematopoietic potential in differentiating ES cells, most frequently using stromal cell lines. Coculture of ES cells on OP9 stromal monolayers demonstrated retention of YS-like kinetics in specification of primitive and definitive progenitors (Nakano et al., 1996; Fujimoto et al., 2003). The reported advantage of stromal cocultures is the potential to quantify output of not only progenitors, but also terminally differentiated cells of different lineages (Zheng et al., 2006). Mostly, stromal coculture systems have been powerful in confirming results initially observed in EB cultures, while achieving a greater degree of homogeneity. Therefore, there is intrinsically increased confidence in cell autonomous vs. non-autonomous effects, uncovering and strengthening observations regarding the importance of micro-environmental cues in hematopoietic potential (Lu et al., 1996; Hidaka et al., 1999; Otani et al., 2004; Zhang et al., 2005; Weisel et al., 2006; Klimchenko et al., 2009).

Controlling stem/progenitor cell output through manipulation of transcription factor networks and key developmental signaling pathways

Early hematopoiesis is tightly regulated, particularly via genetic mechanisms that modulate transcription factor networks, which themselves are subject to influence by major receptor-mediated signaling pathways. Many key factors and pathways have pleiotropic functions in multiple developmental programs, and are essential for appropriate commitment of mesodermal and multipotent precursors. Consequently, early embryonic lethality in transgenic mouse models has made *in vivo* assessments of their roles elusive. However, several groups have exploited the ability to obtain relevant transgenic ES cell lines and examine gene-specific defects *in vitro*. This strategy was first employed to examine the role of GATA factors, a six-member family of transcription factors that binds to eponymous DNA sequences. *Gata1* and stem cell leukemia (*Scl/Tal1*) genes are expressed concomitantly with the first hematopoietic events in YS (Palis et al., 1999). *Gata1* null ES/EBs and OP9 cocultures showed *Gata1* to be required for primitive erythropoiesis (Simon et al., 1992; Suwabe et al., 1998), and for survival of definitive erythroid progenitors past the proerythroblast stage (Weiss et al., 1994). Additionally, conditional re-introduction of *Gata1* rescues associated hematopoietic defects (Zheng et al., 2006). Genetic deletion of *Gata1* is associated with increased expression of *Gata2*,

which is also required for normal YS hematopoiesis (Tsai et al., 1994), suggesting an epistatic relationship between the mesodermally-expressed GATA genes. Deletion of the visceral endoderm marker *Gata4* showed an additional partial dependence on non cell-autonomous instructive signals (Bielinska et al., 1996). Further studies examining the role of *Scl* revealed it to be another essential factor in hematopoietic specification. Genetic deletion of *Scl* impairs YS hematopoiesis (Robb et al., 1995; Shivdasani et al., 1995), and *Scl* null cells only contribute to non-hematopoietic tissues in chimeras (Robb et al., 1996). Experiments examining transcription factor control of primitive hematopoeisis additionally identified rhombotin-like 2 (*Rbtn2/Lmo2*) (Warren et al., 1994), erythroid kruppel-like factor (*Eklf)* (Southwood et al., 1996), and others (Nogueira et al., 2000; Li et al., 2006; Zou et al., 2007) as being required for primitive hematopoiesis in ES/EB cultures. Alternatively, several factors including *Runx1* (Okuda et al., 1996; Miller et al., 2001; Lacaud et al., 2002), the myeloblastosis proto-oncogene family member *c-myb* (Krause et al., 1998; Clarke et al., 2000), and others (Kitajima et al., 1999; Saleque et al., 2002) were shown to be required for normal definitive but not primitive hematopoiesis.

The power of this approach was galvanized upon the advent of inducible ES cell lines. These new lines allowed manipulation of gene expression in a conditional, time-dependent manner to mimic or perturb discrete events during the course of EB differentiation. The most notable platform for this type of study is the AinV cell line developed by Kyba and Daley (Kyba et al., 2002) to induce expression of the homeobox family gene *HoxB4*. Along with several other homeobox factors, *HoxB4* is a potent stimulator of stem/progenitor cell output (Sauvageau et al., 1995; Helgason et al., 1996; Pineault et al., 2002; Lengerke et al., 2007). This cell line employs engineered loci on the X chromosome and chromosome 6 to allow single-site targeted insertion of a transgenic construct, with tetracycline-mediated ("tet-on") transactivation via expression of the reverse tet-transactivator protein from the constitutive ROSA26 promoter. A subsequent study using tamoxifen/estrogen receptor-driven inducible transcription of *HoxB4* confirmed these results and identified a non cell-autonomous role for the *Wingless/Integrase-1* (WNT) signaling pathway (Jackson et al., 2012). Derivative cell lines using AinV as the parental platform have enabled several groups to make important discoveries regarding control of hematopoietic potential during different developmental windows. Transgene expression around day 2 of EB differentiation can be used to gauge effects on specification of mesodermal precursors. Induction on day 4 after hemangioblast specification can be used to assess genetic control of subsequent hematopoietic expansion. This type of strategy has revealed additional roles for *Stat5* (Kyba et al., 2003) and the microRNA *miR-126* (Sturgeon et al., 2012) in stimulating hematopoietic output; and MAX dimerization protein 4 (*Mxd4*) (Boros et al., 2011) and LIM homeobox 2 (*Lhx2*) (Dahl et al., 2008) in inhibiting it.

The AinV inducible system has been advantageous in elucidating the contributions of transforming growth factor-β (TGF-β) family members, particularly through pathway-restricted effects of bone morphogenetic protein (BMP) signaling via mothers against decapentaplegic homolog (SMAD) effector molecules. Our group has shown in AinV lines allowing either conditional expression or shRNA-mediated knockdown, that *Smad1* first promotes expansion of the hemangioblast population from mesodermal precursors (Zafonte et al., 2007), then later restricts pan-hematopoietic potential after hemangioblast specification (Cook et al., 2011). The highly similar *Smad5* functions antagonistically during this stage and specifically promotes primitive erythropoiesis (Liu et al., 2003; McReynolds et al., 2007; Cook and Evans, 2014). Upstream modulation of BMP receptor activation in this system reveals SMAD-independent control through mitogen-activated protein kinase (MAPK) signaling of myeloid progenitor output (Cook and Evans, 2014). Additional studies by Perlingeiro and colleagues have shown a contributing role for the TGF-beta accessory receptor endoglin (*Eng*). Conditional expression of activin receptor-like kinase (*Alk1*) rescues the primitive hematopoietic defect and restores SMAD1 activation impaired in *Eng* null cultures (Zhang et al., 2011). Conditional expression of *Eng* on EB day 2 additionally promotes expression of key hematopoietic marker genes (*Scl*, *Gata1*, *Runx1*) concomitant with SMAD1 activation, in a manner dependent on *Scl* and intact BMP signaling (Baik et al., 2012).

Several studies have explored the hematopoietic roles of additional developmentally important signaling pathways, notably NOTCH and WNT pathways. Notch receptor expression is enriched in hematopoietic tissues (Walker et al., 2001), and is specifically required in embryonic hematopoiesis after the shift to fetal liver as the relevant site (Hadland et al., 2004). Additional experiments utilizing derivatives of the AinV system that allow sorting of reporter-labeled developmental markers have identified antagonistic roles for NOTCH and WNT signaling in limiting and promoting primitive erythropoiesis, respectively (Cheng et al., 2008). However, deletion of the NOTCH pathway ligand Delta-like ligand 4 (*Dll4*) resulted in impairment of BL-CFC specification and subsequent primitive hematopoietic potential (Laranjeiro et al., 2012). Activation of the WNT pathway is specifically required for expansion of hematopoietic progenitors initiated by the hemangioblast (Nostro et al., 2008). The study of these signaling networks has been useful not only to elucidate requirements for biological decisions in a stepwise manner from mesoderm to multipotential progenitors to hematopoietic outgrowth, but also to initiate an evolving toolkit to manipulate those decisions, as will be outlined in the next section.

The next step: how observations in yolk sac hematopoiesis can inform attempts to generate clinically relevant progenitor populations

A crucial factor that contributes to defining transitions between multipotent and successively more fate-restricted progenitors is the VEGF receptor-2, commonly called fetal liver kinase 1 or kinase insert domain receptor (FLK1/KDR). Genetic deletion of *Flk1* results in endothelial but not primitive hematopoietic impairment in chimeric mice (Shalaby et al., 1997). However, subsequent studies in stromal cocultures determined that *Flk1* delivers important instructive cues to generate normal hematopoietic progenitors (Hidaka et al., 1999), and controls fate decisions through differential cell surface expression of CD41 (Otani et al., 2005). VEGF confers dose-dependent survival to primitive progenitors (Martin et al., 2004), and FLK1 marks primitive streak mesoderm predictive of hematopoietic potential (Era et al., 2008). Additional studies have identified markers to differentiate between hemangioblast and hemogenic endothelium, such as cell-surface intercellular adhesion molecule 2 (ICAM2) (Pearson et al., 2010) and the Ets-related protein encoding transcription factor ETV2 (Wareing et al., 2012). Other molecules such as podocalyxin (Zhang et al., 2014) have been shown to distinguish primitive from definitive hematopoietic potentials.

There are inherent limitations to the imposition of artificial culture conditions dependent on serum or on poorly understood cues from other cell types. Efforts to refine approaches to studying and isolating desired progenitor cell types under defined conditions have progressed over several years. The result is an evolving understanding of the relative contributions of Activin/Nodal, BMP, and VEGF to mesoderm-derivative fate decisions (Nostro et al., 2008; Irion et al., 2010; Sturgeon et al., 2014), in combination with reporter-based techniques to sort and manipulate intermediate cell types. Multiple studies have identified the ability of BMP ligands to stimulate formation of hematopoietic ventral mesoderm in serum-free cultures (Johansson and Wiles, 1995; Wiles and Johansson, 1997; Pick et al., 2007). Following this step, there are additional requirements for VEGF and WNT to generate progenitor subsets sequentially demarcated by single or combined expression of Brachyury, FLK1, and forkhead box protein A2 (FOXA2), with WNT signaling specifically active in determining primitive hematopoietic output (Nostro et al., 2008). Moreover, fine-tuning of Activin/VEGF/BMP stimulation can bifurcate populations into early and late FLK1-expressing sub-types. These populations respectively display hemangioblast-like potential for primitive lineages analogous to YS, and hemogenic endothelium-like potential for AA4.1^{+}/CD41^{+} myeloid/lymphoid progenitors (Keller et al., 1993; Ferkowicz et al., 2003; Irion et al., 2010). Directed generation of myeloid/lymphoid progenitors and other definitive sub-types may have important implications for therapeutically-oriented strategies in human cell culture systems (Fig. 1B).

There is great potential to combine these observations with emerging techniques to derive human progenitor cell types from embryonic stem (hES) and induced pluripotent stem (iPS) cell cultures. Culture systems analogous to those described above have been devised to generate hematopoietic progenitors from hES cells (Wang et al., 2005; Grigoriadis et al., 2010), and to distinguish developmentally relevant progenitors (Sturgeon et al., 2014) in both hES and iPS cultures. Recent studies by the Keller laboratory have identified CD235a as a cell-surface marker that distinguishes hemangioblast-like cells with primitive potential from hemogenic endothelium with definitive lymphoid potential. These studies demonstrate that important progenitors can be separated during EB development within a window of responsiveness to Activin/Nodal signaling (Sturgeon et al., 2014). There is additionally a reported balance between retinoic acid and WNT signaling pathways in controlling hematopoietic stem cell output from hemogenic endothelium marked by expression of FLK1 and SRY box-17 (SOX17) (Chanda et al., 2013; Clarke et al., 2013). Such observations encourage continuing efforts to optimize directed differentiation protocols. Finally, additional recent investigations into transcription factor-mediated direct conversion of murine fibroblasts to hemogenic endothelium-like precursors (Pereira et al., 2013) and of human endothelial cells to multipotent progenitors (Sandler et al., 2014) hold promise as alternative platforms that may in some instances bypass the requirement for pluripotent cell lines altogether. In conclusion, modeling hematopoietic ontogeny with murine embryonic stem cells and their derivatives has established a foundation from which greater understanding of regulatory controls of stem and progenitor cell output is constantly emerging. These efforts continue to drive evolving strategies to manipulate the production of developmentally and clinically relevant cells of the primitive and definitive lineages.

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Compliance with ethics guidelines

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