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PERSPECTIVES

Follistatin as potential therapeutic target in prostate cancer

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Abstract Follistatin is a single-chain glycosylated protein whose primary function consists in binding and neutralizing some members of the transforming growth factor-β superfamily such as activin and bone morphogenic proteins. Emerging evidence indicates that this molecule may also play a role in the malignant progression of several human tumors including prostate cancer. In particular, recent findings suggest that, in this tumor, follistatin may also contribute to the formation of bone metastasis through multiple mechanisms, some of which are not related to its specific activin or bone morphogenic proteins' inhibitory activity. This review provides insight into the most recent advances in understanding the role of follistatin in the prostate cancer progression and discusses the clinical and therapeutic implications related to these findings.

Keywords Activin · Bone metastasis · Cancer · Follistatin · Prostate cancer

Introduction

Prostate cancer (PCa) is the third most common cause of death from cancer in men of all ages and the most common cause of death from cancer in men over 75 years old [1, 2]. The number of affected subjects is expected to increase as the population of males over the age of 50 grows worldwide [1, 2]. Complete surgical resection or radiotherapy is currently the only potentially curative treatment for patients with localized prostate cancer. However, about one third of these patients relapse after radical prostatectomy due to undetected metastatic disease [3]. The lack of effective clinical treatments for prostate cancer reflects, in part, the incomplete knowledge of the molecular mechanisms involved in the development and progression of this tumor [4]. Therefore, a better understanding of this process may lead to the identification of new molecular targets and more effective therapeutic options in PCa treatment. In this scenario, emerging evidence indicates that activin (Act), a member of the transforming growth beta (TGF-β) superfamily of growth factors, appears to play a role in the malignant progression of prostate cancer [5–7]. This hypothesis is supported by growing experimental and clinical observations which highlight that the activin signaling pathway is deregulated in PCa and that this phenomenon is associated with the onset of more aggressive forms of this tumor [6, 7]. The deregulation of the activin signaling pathway appears to be the result of several mechanisms including alterations in the expression level of some endogenous inhibitors of activin such as follistatin (FLS) [6, 7]. Therefore, this inhibitor may be of potential clinical interest as a novel molecular target in the treatment of prostate cancer. The aims of the present paper are to provide an updated systematic review on the role of FLS in prostate cancer progression and to discuss the clinical and therapeutic implications of these novel findings.

Follistatin: structure and functions

Follistatin is a cystein-rich glycosylated polypeptide chain of 31–39 kDa that binds to Act with high affinity (850–500 pM) [8, 9]. This inhibitor was originally isolated from porcine ovarian follicular fluid and identified as a molecule implicated in the regulation of the secretion of follicle-stimulating hormone [8, 9]. FLS is a product of a single

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gene, located on the long arm chromosome 5q11.2. Sequence analysis demonstrated that the *FLS* gene consists of six exons separated by five introns transcribed into the precursor FLS317 and FLS344 messenger RNA (mRNA) forming at least two molecular weight forms by alternate splicing, i.e., FLS288 (from pre-FLS317) and FLS315 (from pre-FLS344) [10] (http://www.ncbi.nlm.nih.gov/LocusLink/s). These isoforms contain a N-terminal domain and three domains, known as follistatin domains (FSD1, FDS2, and FDS3 respectively), which differ in their amino acid composition [8, 10]. Each FLS domain consists of 73–77 amino acids and is distinguished by ten conserved cysteine residues. In particular, FSD1 contains an amino acid sequence known as a heparin binding sequence (HBS) that enables FLS to bind with high-affinity cell surface proteoglycans [10, 11]. The heparin binding sequence of follistatin has been mapped to a lysine- and arginine-rich sequence within residues 75–86 in FSD1 [11]. Experimental in vivo studies show that FLS knockout mice dies soon after birth due to a variety of skeletal and cutaneous defects [12]. The transcription of *FLS* gene may be stimulated by Act, TGF-β, forkhead domain transcription factor L2 (FoxL2) via Smad proteins [13, 14], gonadotropin-releasing hormone [15], GLI2 (a transcription factor activated by hedgehog signaling) [16], dexamethasone [17], androgens [18], activators of Wnt signaling [19, 20] and 1,25-dihydroxyvitamin D (1,25(OH)2D3) [21]. Conversely, *FLS* gene expression has been shown to be downregulated, according to the cell type, by peroxisome proliferator-activated receptor gamma (PPRA-γ) or the transcriptor factor epiprofin, also known as Sp6 [22, 23]. The FLS288 and FLS315 splice variant isoforms are the most common ones. They are widely present and differentially expressed in human tissues [8–10, 24, 25]. The longer FLS315 variant is predominant, while the FLS288 isoform accounts for less than 5 % of the encoded mRNA [26, 27]. A third intermediate form, namely FS303, is presumably derived by proteolytic processing of the C-terminal domain of the FS315 variant [10, 26, 28]. Both FLS315 and FLS288 bind to Act with high affinity [9, 26, 28–32]. However, while FLS288 binds heparan sulfate proteoglycans with high affinity and may function as local regulator of Act [33], the longer FLS315 isoform, which is the main circulating form of this molecule, does not bind to the cell surface proteoglycans as it contains a tail consisting in an acidic extension at the C terminus of the 27 amino acid residues that can mask the heparin binding sequence of follistatin when the molecule is in an unbound state [9, 26, 31–33]. After binding to Act, the tail region unmasks the heparin binding sequence and the Act–FS315 complex can then bind to the cell surface proteoglycans [26, 30, 33]. This phenomenon may explain the reason why FLS315 is secreted faster than FLS288 and why FLS315 may enter the circulation in a larger fraction [24]. Within the circulation, 70–90 % of FLS315 exists in the bound form [34]. The interaction between a molecule of FLS and a molecule of Act results in an almost irreversible binding complex that prevents Act to interact with its specific receptors (Fig. 1). This phenomenon ultimately leads to the inhibition of the biological effects induced by Act on various cells and tissues [8, 9, 28, 29, 31] (Fig. 1). Moreover, the irreversible binding of FLS to Act facilitates its internalization and the subsequent degradation of this complex by the lysosomal enzymes [8, 9, 29] (Fig. 1). The Act–FLS binding complex is generally composed of one Act and two FLS molecules [29] (Fig. 1). However, the exact function of the bound form remains to be defined. On the other hand, the FS315 does not appear to function as serum carrier for Act as its binding to this growth factor appears to be irreversible [9,10, 26, 28, 29, 31]. FLS may also bind with lower affinity to other members including bone morphogenic proteins (BMPs), in particular, BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-11 [34–37], myostatin [38] and TGFß3 [39]. Additionally, FLS may also interact with two molecules that are not related to the TGF-β family, namely, the serum pan-protease inhibitor α2-macroglobulin [40] and the pro-angiogenic factor angiogenin [41]. However, the functional significance of the interactions between FLS and these molecules remains to be fully elucidated.

Follistatin's role in the physiological growth and function of prostate gland

FLS is widely distributed in adult tissues including the prostate where it is usually co-localized with activin subunits and/or activin receptors [5, 8, 9, 25, 34, 42–45]. Compelling evidence indicates that the FLS/Act system plays a role in the paracrine/autocrine control of the physiological growth and functions of several tissues including the prostate gland [7, 42, 46–59]. The hypothesis that the interplay between FLS and Act may contribute to regulate prostate tissue homeostasis is suggested by the findings that FLS and Act receptors are predominantly co-localized in the developing prostatic epithelium [45–47]. Furthermore, this molecule is expressed throughout the epithelium of developing prostate and maintained into the mature glands [48]. In particular, immunohistochemical studies highlight a staining of FLS mainly in the stroma cells, while Act is mainly detected on the epithelial cells [46, 47]. Interestingly, the prostate epithelium and stroma differentially produce FS288 and FS315 isoforms respectively, thus suggesting a correlation between a specific isoform of FLS expression and cell type [43]. On the other hand, in vitro studies by Wang et al. [42] show that fresh human primary tissues and primary culture of human epithelial prostate cells actively secrete both Act and FLS that exert opposite effects, i.e.,

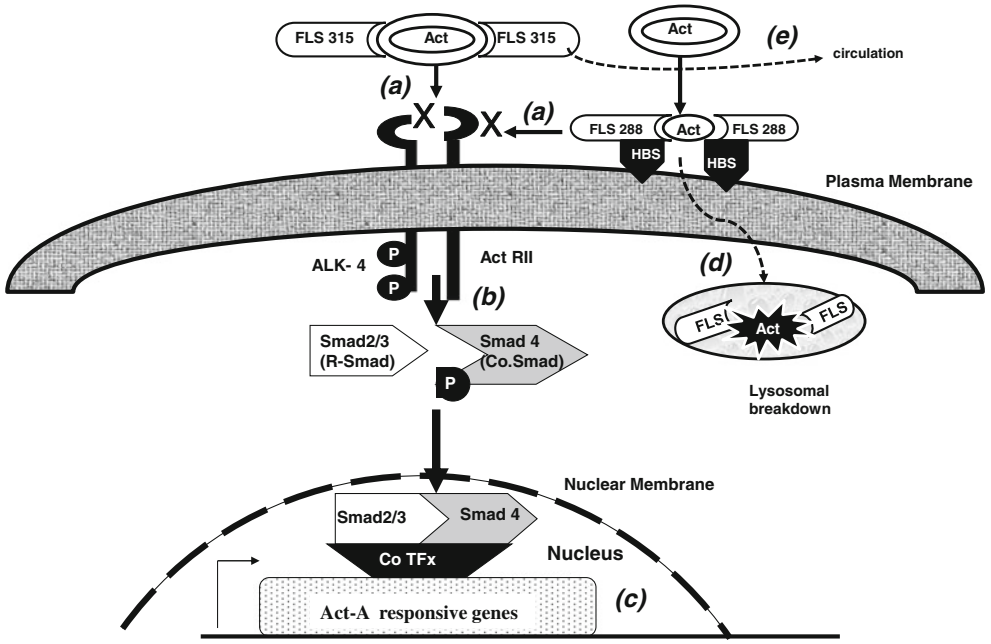

Fig. 1 Schematic representation of the activin–follistatin interactions and the resulting neutralization pathway. (***a***) FLS315 and FLS288 bind to Act with high affinity. The Act–FLS binding complex is composed of one Act and two FLS molecules. (**a**) Free Act dimers associate with membrane-bound FLS 288 which has a strong affinity for cell surface heparan sulfate proteoglycans (HSPG) (***a***). (**b**) The binding of FLS to Act blocks the interactions between Act and its receptors (Act type II receptor). (**c**) This phenomenon, ultimately inhibits the activation of activin mediated downstream signal transduction pathways and, consequently, the biological effects induced by this growth factor on various cells. (***d***) The irreversible binding of FLS to Act facilitates its internalization and the subsequent degradation of this complex by the lysosomal enzymes. (**e**) FLS315, which is the main circulating form of this molecule, exhibits limited binding to HSPG. The fate of these complexes and the function of this pathway are unclear. However it is currently suggested that circulating FLS315 bound to Act to facilitate its clearance and/or prevent the diffusion of this cytokine from its local site of action

inhibiting or promoting effects respectively, on the growth of normal prostatic epithelial cells. These findings are consistent with the observations that FLS may act as a positive regulator of branching morphogenesis of the prostate while Act may function as a negative regulator of this phenomenon [43, 46–48]. The specific role of FLS in the promotion of the branching morphogenesis is further supported by the findings that in newborn prostate explants grown in the absence of testosterone, the addition of FLS increases the growth and branching of the prostate gland [47, 48]. These studies further stress the concept that a balanced interaction between FLS and Act is an essential prerequisite in order to regulate the prostate ductal growth and branching morphogenesis.

Follistatin in prostate cancer progression

The process of prostate organogenesis ultimately leads to the development of a mature gland composed of both highly differentiated contractile muscle cells and secretory epithelial cells [7, 47, 48]. Experimental evidence shows that, in physiological conditions, reciprocal homeostatic interactions between smooth muscle and epithelial cells contribute to regulate cell growth quiescence and functional differentiation [46–48]. However, perturbations of these cell–cell interactions may cause loss of control of the epithelial cell and muscle cell growth and differentiation and, eventually, may trigger the process of malignant transformation [47–51]. As the FLS/Act system appears to have a role in the regulation of prostate growth and development, it is conceivable to hypothesize that a deregulation of this system may cause alterations of the normal homeostasis of prostate tissue and may contribute to the development and progression of prostate cancer [5–7, 46–48]. In support of this hypothesis, in vitro studies on human LNCaP androgen-sensitive prostate cancer cells highlight that FLS expression increases during the progression of prostate cancer toward more malignant androgen-independent disease variants [40, 52–54]. Furthermore, other in vitro observations show that unlike LNCaP cancer cells which are responsive to the growth inhibitory effect of Act, androgen-independent PC3 human prostate cancer cells which, conversely, are resistant to the growth-promoting effects of Act actively secrete FLS [7, 37, 40, 44]. In particular, PC3 tumor cells predominantly express the FS288 isoform while LNCaP or DU145 tumor cells mainly express the FS315 variant [54, 55]. These findings are in line with some data from in vitro studies which highlight that FLS288 expression in PC3 tumor cells appears to be specifically associated with their resistance to the growth-inhibiting effects of Act [22, 32, 40, 43]. Additional evidence in support of the specific involvement of FLS288 in this

phenomenon is provided by the findings that the inhibitory activity of Act on LNCaP and DU145 cells can be counteracted by the addition of a tenfold excess of FLS288, but not by the addition of inhibin, another intracellular inhibitor of Act [7, 9, 42, 44, 53]. The preferential inhibiting effect of the FLS288 variant form on Act may be, in part, explained by the fact that this inhibitor is endowed with a neutralizing effect on Act greater than that of the FLS315 isoform and that FLS288, rather than FLS315, appears to be involved in the inactivation and clearance of activin [8, 26, 28, 29, 31] (Fig. 1). On the other hand, the growth-promoting effects of FLS on the prostate cancer cells are also indirectly proven by in vitro experiments which show that some plant-derived polyphenolic compounds with estrogen-like properties such as soy isoflavones may inhibit the prostate cancer cell growth by downregulating several genes involved in tumor cell proliferation, angiogenesis and metastasis, including *FLS* gene [56]. Furthermore, van der Poel et al. [57] also show that rapamycin, a specific inhibitor of the mammalian serine/threonine protein kinase mammalian target of rapamycin (mTOR), inhibits PC3 prostate cancer cells proliferation by causing cell cycle arrest in G1 phase and that this phenomenon is associated with an increase in the expression levels of BMP-4 and a decrease in the levels of FLS. Intriguingly, recent studies by Havard et al. [58] highlight that Act may promote in vitro the entry of PCa cells in a dormant state caused by a slight increase of the osmotic pressure in culture medium. On the basis of these findings, it should be conceivable to speculate that an increase in the expression level of FLS may facilitate the escape of tumor cells from a dormant state regulated by Act thus fostering the growth and dissemination of these cells. Finally, Ye et al. [59] show that the loss of endogenous BMP-7 in the prostate cancer cells is associated with an increased invasiveness and motility which appears to be facilitated by alterations in the expression level of some BMPs antagonists including FLS. Although these studies indicate that the mechanisms through which FLS may promote the malignant progression of PCa appears to be essentially correlated to its Act and or BMP inhibitory activity, accumulating evidence indicates that this molecule may also contribute to promote PCa progression by additional mechanisms not related to its Act or BMP inhibitory activity. For instance, recent observations by Gao et al. [60] show that FLS may be up-regulated and translocated to the nucleoli in the HeLa cells in response to glucose deprivation. The overexpression of FLS, in turn, negatively regulates rRNA synthesis and ribosome biogenesis. These effects are known to delay glucose deprivation-induced apoptosis [61]. Conversely, a downregulation of FLS elicits opposite effects [60]. As the increased resistance of cancer cells to glucose deficiency contributes positively to tumor progression [61], it is reasonable to hypothesize that FLS, through these mechanisms, could indirectly foster tumor development. On the other hand, these data further confirm the previous findings of the same authors [41] highlighting that in HeLa cells, FLS is present in the nucleus and may interact with angiogenin, a 14-kDa protein endowed with pro-angiogenic functions, including endothelial cell activation, which may also promote migration, invasion, proliferation, and formation of tubular structure [62–64]. Finally, FLS has been reported to bind to type III TGF-β [39]. As this isoform appears to play a major role in regulating the inhibition of PCa growth [65], it should be conceivable to speculate that FLS might probably also promote the malignant progression of PCa by interacting with type III TGF-β.

Follistatin and tumor angiogenesis

Growing experimental observations indicate that FLS may be also involved in the regulation of tumor angiogenesis thereby fostering the malignant progression of prostate cancer. In this context, early studies of Kozian et al. [66] showed that FLS may promote in vitro the proliferation of human umbilical vein endothelial cells, while, in vivo, this molecule is moderately angiogenic. These findings are consistent with some in vitro observations showing that FLS is upregulated in proliferating human microvascular endothelial cells (MVEC), but not in tubular MVEC [67]. Moreover, Gao et al. [41] have identified FLS as a binding partner of angiogenin which is a potent stimulator of angiogenesis and tumor cell proliferation [68]. Furthermore, other in vitro observations highlight that FLS, in concert with vascular endothelial growth factor (VEGF), appears to facilitate the formation of new blood vessels by stimulating the production of matrix metalloproteinase-2 (MMP-2), a proteolytic enzyme implicated in tumor angiogenesis and bone metastasis formation in prostate cancer [69–71]. On the other hand, the hypothesis of a possible involvement of FLS on tumor angiogenesis is further corroborated by in vivo studies of Krneta et al. [72] who have recently described that FLS may function as a potent angiogenesis stimulator also in severe combined immunodeficiency (SCID) mice transplanted with R30C human mammary tumor cells. These findings fit well with other recent observations which highlight that potent antiangiogenic molecules present in dietary sources, such as derived polyphenolic compounds with estrogen-like properties, may inhibit PCa cells growth by downregulating the expression of several genes involved in the malignant progression of this tumor, including *FLS* [56, 73]. However, unlike these findings, Ogino et al. [73] reported that, in NK cell-depleted SCID mice, the transfection of *FLS* gene resulted in the suppression of the experimental multiple organ metastases due to the inhibition of angiogenesis by small-cell lung cancer cells. The discrepancies in these results are, in part, currently explained through the use of different animal tumor models and the possible different role of the Act/FLS system at different stages of tumor progression and in the malignant progression of the different types of tumors [72, 74–79].

Follistatin and metastasis

The possible involvement of FLS in tumor angiogenesis supports the concept that this molecule may also facilitate the dissemination of tumor cells to distant organs via the angiogenic route. In line with this hypothesis, emerging evidence suggests that, at least in some tumors, FLS appears to facilitate the metastatic process [7, 72, 80–85]. On the other hand, a possible involvement of FLS in the pathogenesis of bone metastasis is not surprising as this molecule has been shown to actively cooperate with activin A in the regulation of normal bone homeostasis [86–89]. Consequently, it is conceivable that a deregulation of FLS expression may eventually result in pathological alterations of the normal bone remodeling processes such as it occurs in metastatic bone diseases. This hypothesis is sustained by some our previous clinical studies that show a significant correlation between circulating levels of FLS and PCa progression [84]. These findings are also consistent with the results from other immunohistochemical and molecular biology observations that highlight a positive correlation between altered expression level of FLS in prostate cancer and a more aggressive behavior of this tumor [7, 51, 52, 85, 90]. The possible mechanisms behind the promoting activity of FLS of PCa bone metastasis formation remain currently to be unraveled. Nonetheless, recent in vitro studies suggest that this molecule, in addition to its tumor angiogenesis promoting activity, may also indirectly facilitate bone metastasis by modulating the adhesion and invasion of tumor cells via BMP-2, BMP-4, and BMP-7, which appear to play a key role in the formation of osteoblastic lesions associated with prostate cancer metastases [59, 90–93]. Interestingly, Simon et al. [94] have recently reported that FLS may indirectly promote tumor cell detachment and migration by up-regulating the expression levels of the enzyme A -Disintegrin and Metalloproteinase-15 (ADAM-15), a disintegrin which cleaves integrin molecules and whose altered expression has been shown to support tumor growth, endothelial interaction, and metastasis of prostate cancer cells [66, 91, 95]. Intriguingly, this latter mechanism has been described so far only for human prostate cancer and neuroblastoma cells [94, 96]. These findings are promising for the discovery of more selective and effective therapeutic strategies in the treatment of prostate cancer.

Follistatin as a marker of prostate cancer progression

The observations that FLS expression levels are altered in tumor tissues [7, 45, 52, 55, 87] and/or in body fluids [83, 84, 93, 94] of patients with prostate cancer are suggestive of a possible clinical usefulness of this inhibitor as an additional marker in the clinical management of these patients. However, studies directed toward this aims are still scanty. Nonetheless, some of our recent clinical observations highlight that FLS serum levels are significantly increased in patients with prostate cancer as compared to those determined in patients with benign prostate hyperplasia or healthy subjects [85]. These findings further confirm previous observations of Sardana et al. [84]. In addition, our investigations show a close relationship between FLS serum concentrations and the presence of bone metastasis or increased PSA levels in these patients and that the ratio between the serum concentration of FLS and Act in PCa patients significantly differs from that measured in normal subjects [85]. These results further suggest a possible relationship between a deregulation of the Act/FLS system and prostate cancer growth and progression, and a close relationship between FLS serum concentrations and the presence of bone metastasis or increased PSA levels in these patients. However, these studies additionally show that, in our series of patients, the diagnostic performance of FLS, as assessed by receiver operating characteristic curve, is not significantly different from that observed for PSA or Activin [85, 97]. Moreover, the combination of FLS with PSA and/or Act does not result in an improved diagnostic accuracy as compared to that determined for each single molecule [84, 85, 97]. Although these findings appear to rule out a diagnostic usefulness of FLS in PCa patients, they indicate that this molecule may be of potential clinical interest as additional circulating marker for the therapeutic management and follow-up of PCa patients [84, 85, 94, 96]. Further

Table 1 Possible mechanisms not related to the Act or BMP inhibitory activity by which FLS may facilitate prostate cancer cell proliferation invasion and metastasis

Mechanism	Reference
Delay of glucose deprivation-induced apoptosis	[60]
Stimulation of endothelial cell proliferation	[66]
Nuclear translocation and interactions with angiogenin	[41]
Stimulation of matrix metalloproteinase-2	[69]
Stimulation of sprouting angiogenesis and VEGF expression	[72]
Up-regulation of disintegrin ADAM-15	[94]
Modulation of adhesion and invasion of tumor cells	[90]

studies with a wider number of subjects may better define the clinical role of FLS in prostate cancer.

Conclusions

There is an increasing evidence that follistatin, an inhibitor of the pleiotropic cytokine of the TGF β superfamily Activin, may be implicated in the malignant progression of prostate cancer [5, 7, 52, 54–56, 83, 88]. Moreover, experimental and clinical observations indicate that FLS may foster the dissemination of tumor cell into distant organs [7, 88]. In particular, recent findings indicate that in PCa FLS may facilitate bone metastasis formation through multiple mechanisms, some of which are independent from its Act or BMPs' inhibitory activity [7, 60, 64, 68, 71, 93] (Table 1). The possible implication of FLS in facilitating bone metastasis formation in patients with prostate cancer is further suggested by some our recent clinical observations that highlight a positive correlation between altered expression levels of FLS and prostate cancer growth and/or presence of bone metastasis [83, 84]. Moreover, recent findings show that FLS may up-regulate ADAM-15, a disintegrin whose altered expression has been shown to specifically facilitate growth, endothelial interaction, and metastasis of prostate cancer cells and neroblastoma cells [66, 91, 94, 95]. These findings make FLS an attractive target for novel therapeutic options in the prevention and treatment of prostate cancer [56, 57, 72] and a potentially useful biomarker in the clinical management of patients with this tumor [83, 84, 92–94, 96]. In support of these hypotheses, recent in vitro experiments report that plant-derived polyphenolic compounds, namely, soy isoflavones, may inhibit prostate cancer cell growth and that this therapeutic effect is also correlated with a downregulation of the *FLS* gene [56]. Similarly, rapamycin, a specific inhibitor of the mammalian serine/threonine protein kinase mTOR, inhibits PC3 prostate cancer cells proliferation by causing cell cycle arrest in the G1 phase, a phenomenon which is associated with a decrease of FLS t levels in this case too [57]. These findings warrant further investigations in order to better assess the clinical role of FLS in prostate cancer.

Conflict of interest The authors declare no conflicts of interest.

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