

## Heart Myofibrillogenesis Occurs in Isolated Chick Posterior Blastoderm: A Culture Model

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Early cardiogenesis including myofibrillogenesis is a critical event during development. Recently we showed that prospective cardiomyocytes reside in the posterior lateral blastoderm in the chick embryo. Here we cultured the posterior region of the chick blastoderm in serum-free medium and observed the process of myofibrillogenesis by immunohistochemistry. After 48 hours, explants expressed sarcomeric proteins (sarcomeric  $\alpha$ -actinin, 61%; smooth muscle  $\alpha$ -actin, 95%; Z-line titin, 56%; sarcomeric myosin, 48%); however, they did not yet show a mature striation. After 72 hours, more than 92% of explants expressed I-Z-I proteins, which were incorporated into the striation in 75% of explants or more (sarcomeric  $\alpha$ -actinin, 75%; smooth muscle  $\alpha$ -actin, 81%; Z-line titin, 83%). Sarcomeric myosin was expressed in 63% of explants and incorporated into A-bands in 37%. The percentage incidence of expression or striation of I-Z-I proteins was significantly higher than that of sarcomeric myosin. Results suggested that the nascent I-Z-I components appeared to be generated independently of A-bands in the cultured posterior blastoderm, and that the process of myofibrillogenesis observed in our culture model faithfully reflected that *in vivo*. Our blastoderm culture model appeared to be useful to investigate the mechanisms regulating the early cardiogenesis.

**Key words:** myofibrillogenesis, early cardiogenesis, blastoderm, chick embryo

### I. Introduction

In early chick cardiogenesis, prospective heart cells are located in posterior lateral region of the epiblast, but not in central to anterior epiblast [5, 14, 23]. During gastrulation, the prospective heart cells migrate into the anterior half of the primitive streak, subsequently leave the primitive streak and accumulate in the anterior region of the lateral plate mesoderm, called the precardiac mesoderm [11]. The anterior lateral plate mesoderm then divides dorsoventrally into the somatic and the visceral mesoderm. The left and right visceral mesoderm migrate anterior midline of the intestinal

portal and fuse to form the primitive heart tube. At this stage (late gastrula to neurula, Hamburger and Hamilton [HH]-stage 6–10) [13], myofibrillogenesis occurs rapidly and the heart starts to beat spontaneously [15].

The processes of *de novo* myofibrillogenesis are highly conserved in vertebrate striated muscle including nascent cardiomyocytes, and so investigators have tried to explain the process involved in the formation of the sarcomeric structure, which consists of an organized I-Z-I component and A-band [26]. Several models have been proposed to explain how myofibrillogenesis is carried out during cardiac muscle differentiation using neonatal/fetal cardiomyocyte culture systems, in which isolated cardiomyocytes lose their mature myofibrils and reorganize myofibrils during cultivation. It has been reported that the first step involves a stress-fiber-like structure as a scaffold, then the I-Z-I proteins (the Z-disk components being sarcomeric  $\alpha$ -actinin,  $\alpha$ -actin

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and Z-line titin) and thick A-bands (consisting mainly of myosin) are assembled independently to later become incorporated into myofibrils [16]. At the onset of myofibrillogenesis, the stress-fiber-like structure consists of mini-sarcomeres containing actin filaments,  $\alpha$ -actinin and non-muscle myosin. Subsequently, the distance between the dense materials of the nascent myofibril increases, titin is incorporated, and non-muscle myosin is replaced by muscle-specific myosin to complete the mature myofibrils [19, 30]. *In vivo* observations of myofibrillogenesis in chick embryonic heart showed that the myofibrillogenesis occurs in a similar manner to that observed in culture models [8].

Despite many studies, there is still uncertainty about the morphological and molecular mechanisms that are required for early cardiogenesis, such as heart mesoderm formation, heart specification and terminal differentiation. To establish a culture system for investigation of the early cardiomyogenesis, we cultured chick posterior blastoderm in serum-free defined medium and observed the process of myofibrillogenesis by immunohistochemistry.

## II. Materials and Methods

### Culture procedures

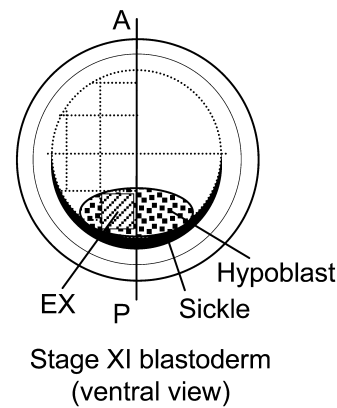
Eyal-Giladi and Kochav (EK)-stage X–XI blastoderms (incubation time 0 hr) [10] were collected on ice-cooled phosphate-buffered saline (PBS), and the posterior blastoderm including the epiblast and associated hypoblast, but not sickle, was isolated using a sharp tungsten needle (hatched square in Fig. 1). The resulting explants were explanted onto chamber slides (Nunc) and cultured in a serum-free defined medium (75% DMEM, 25% McCoy's medium, supplemented with  $10^{-7}$  M dexamethasone and penicillin-streptomycin, Sigma, St. Louis, MO, USA) [18].

### Indirect immunofluorescence microscopy

Immunohistochemistry was performed, as described elsewhere [24]. Cultures were drained of medium, fixed with 4% paraformaldehyde/PBS for 30 min at room temperature, and rinsed with PBS. Specimens were blocked for 1 hr with 1% BSA (bovine serum albumin) containing 0.1% Triton X-100, and then incubated with primary antibody mixture at 4°C overnight. They were then rinsed with PBS and incubated with FITC- or RITC-conjugated secondary antibody mixture for 1 hr at room temperature. Samples were observed under a laser confocal microscope (Zeiss). Explants that expressed sarcomeric proteins or generated mature striations were counted under a microscope. Percentage incidence of sarcomeric protein- or striation-positive explants was calculated. Statistical analyses were performed using Fisher's exact test and Bonferroni's correction for multiple comparison. Significance level was less than 5%.

### Antibodies

The monoclonal antibodies anti-Z-line titin (anti-titin, clone 9D10, IgM, supernatant), anti-sarcomeric myosin heavy chain (clone MF20, IgG2b, supernatant) [3] and anti-

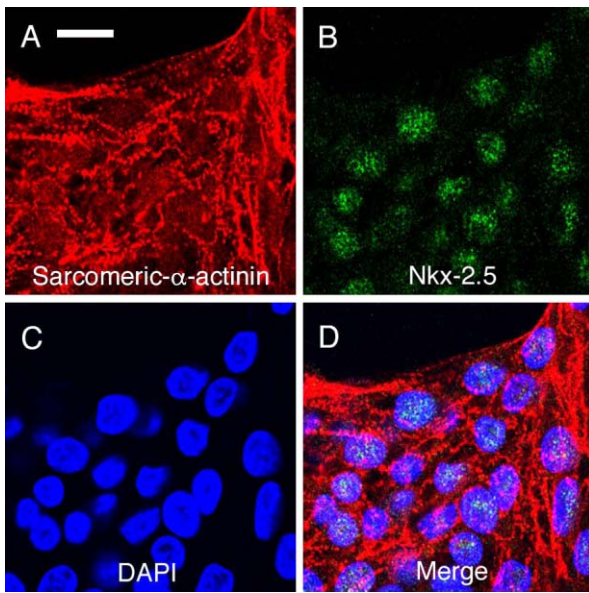


**Fig. 1.** Explant from prestreak chick blastoderm. EK-stage X–XI blastoderms were collected. Posterior regions (hatched square) containing epiblast and hypoblast were isolated and cultured in serum-free medium. Note that the EK-stage indicates the embryonic stages before gastrulation by Eyal-Giladi and Kochav (1976) [10]. A, anterior; P, posterior.

skeletal muscle sarcoplasmic reticulum (clone 12/101, IgG1, supernatant) [12] were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). The monoclonal antibodies, anti-smooth muscle  $\alpha$ -actin (SMA, clone 1A4, IgG2a, 1:400) [28] and anti-sarcomeric  $\alpha$ -actinin (clone EA53, IgG1, 1:600), were purchased from Sigma (St. Louis, MO, USA). Anti-Nkx2.5 (goat IgG, 1:100) was purchased from Santa Cruz Biotechnology (Santa Cruz, Ca, USA). For double immunohistochemistry, we used FITC-conjugated goat anti-mouse IgG2a, RITC-conjugated goat anti-mouse IgG2a, FITC-conjugated goat anti-mouse IgG1, FITC-conjugated goat anti-mouse IgG2b, RITC-conjugated goat anti-mouse IgM (Southern Biotechnology, Birmingham, AL, USA) and FITC-conjugated donkey anti-goat IgG (Chemicon, Temecula, CA, USA) as secondary antibodies. Secondary antibodies we used were diluted 1:100 and did not cross-react with chick embryonic tissue (data not shown).

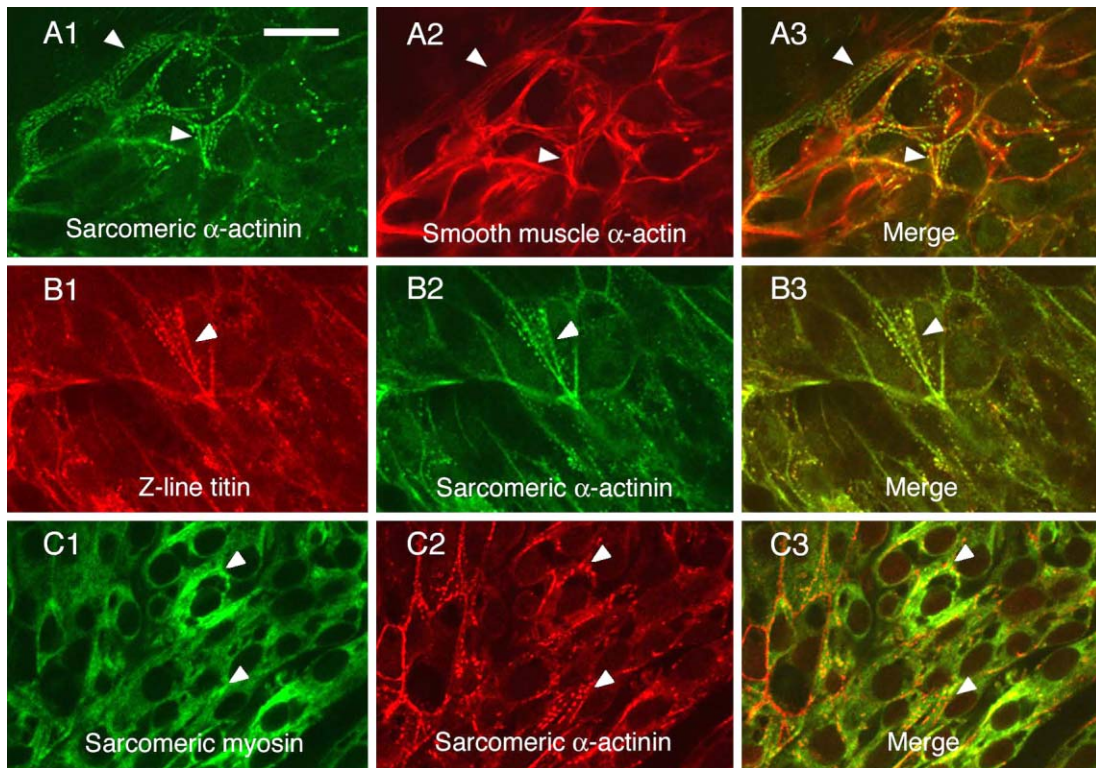
## III. Results

Prospective heart-forming posterior blastoderm from EK-stage X–XI embryos (hatched region in Fig. 1) was cultured in serum-free defined medium, fixed and doubly stained with antibodies against various cardiomyocyte-specific proteins, such as Nkx-2.5, smooth muscle  $\alpha$ -actin (SMA), sarcomeric  $\alpha$ -actinin, Z-line titin and sarcomeric myosin. It has been reported that SMA is initially expressed in precardiac mesoderm at HH-stage 6 (late gastrula) and then recruited as an initial heart  $\alpha$ -actin [6, 29]. After 24 hr in culture, there was no detectable staining for these heart-specific proteins (data not shown). After 48 hr in culture, the heart-specific transcription factor Nkx-2.5 was detectable in the nuclei of cells that were expressing sarcomeric  $\alpha$ -actinin (Fig. 2) [17]. At this time, tiny bead-like deposits of sarcomeric  $\alpha$ -actinin were observed (61% of explants, n=28)



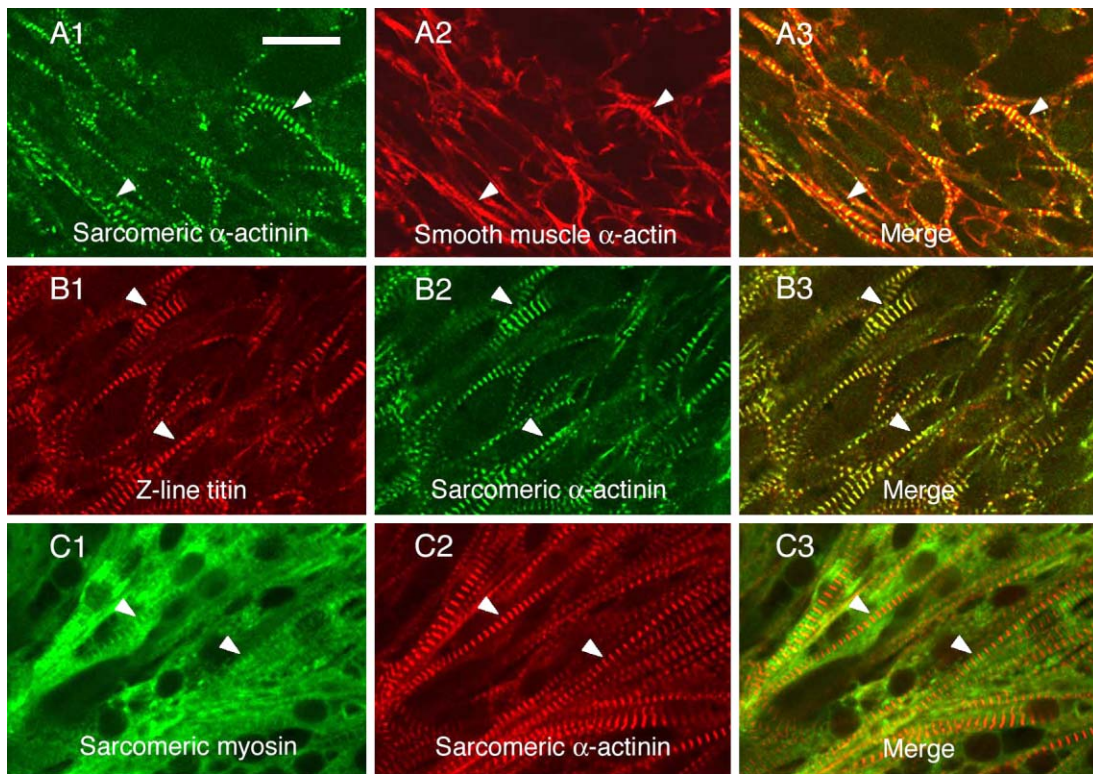
**Fig. 2.** Cultured posterior blastoderm expresses the heart-specific transcription factor Nkx-2.5. After 48 hr in culture, explants were fixed and stained with antibodies against heart specific transcription factor (Nkx-2.5) and early heart sarcomeric protein (sarcomeric  $\alpha$ -actinin). Cells that were expressing sarcomeric  $\alpha$ -actinin (A) expressed Nkx-2.5 (B) in their nuclei (C, D). Bar=20  $\mu$ m.

along the SMA-positive (95%, n=19) filaments, which were terminating at epithelial-cell borders (Figs. 3A, 5). Sarcomeric  $\alpha$ -actinin and SMA were also present around the circumference at the cell-cell border. At this time, no apparent mature sarcomeric structure consisting of sarcomeric  $\alpha$ -actinin and SMA was observed (Fig. 5). The amino terminal domain of titin (Z-line titin) was expressed (56%, n=18) and co-localized with the bead-like deposit of sarcomeric  $\alpha$ -actinin (Figs. 3B, 5). Sarcomeric myosin was distributed in a diffuse manner (46%, n=28) in cells within which bead-like sarcomeric  $\alpha$ -actinin was observed (Figs. 3C, 5). After 72 hr in culture, 92% or more of explants expressed sarcomeric  $\alpha$ -actinin (92%, n=64), SMA (100%, n=37) and Z-line titin (94%, n=36). Sarcomeric myosin was expressed in 63% of explants (n=49). At this time, 75% or more of explants exhibited sarcomeric staining of sarcomeric  $\alpha$ -actinin (75%), SMA (81%) and Z-line titin (83%). By contrast, A-band associated sarcomeric myosin was exhibited in 37% of explants (Figs. 4, 5). Statistical analysis (Fisher's exact test) showed that there was significant difference in the percentage incidence of expression or striation among four sarcomeric proteins such as sarcomeric  $\alpha$ -actinin, SMA, Z-line titin and myosin. The results of each individual pairwise comparison are indicated in Figure 5. There was no significant difference in the incidence of expression or striation among I-Z-I proteins, and the incidence of expres-

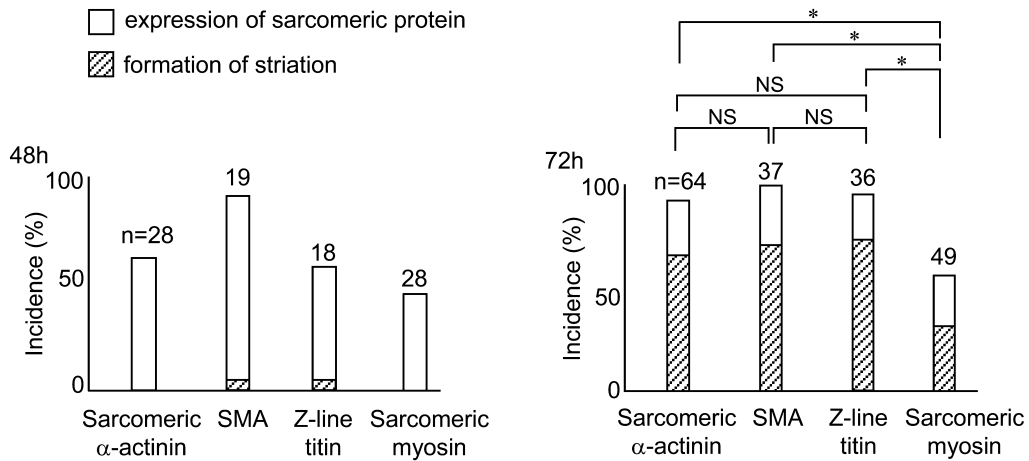


**Fig. 3.** EK-stage X–XI posterior blastoderm generates premature myofibrils after 48 hr in culture. Tiny bead-like deposits of sarcomeric  $\alpha$ -actinin (arrowheads in A1) appeared along filamentous smooth muscle  $\alpha$ -actin (arrowheads in A2–3). Z-line titin was also seen as tiny bead-like deposits and co-localized with the sarcomeric  $\alpha$ -actinin (arrowheads in B1–3). Sarcomeric myosin exhibited a diffuse cytoplasmic staining (C1) and was distributed independently of the bead-like deposits of sarcomeric  $\alpha$ -actinin (arrowheads in C2 and C3). Bar=20  $\mu$ m.





**Fig. 4.** EK-stage X–XI posterior blastoderm generates mature myofibrils after 72 hr in culture. Sarcomeric  $\alpha$ -actinin (A1) and smooth muscle  $\alpha$ -actinin (A2) were incorporated into mature I-Z-I structures (arrowheads in A1–3). Z-line titin was co-localized with striated sarcomeric  $\alpha$ -actinin (arrowheads in B1–3). Sarcomeric myosin was also incorporated into nascent sarcomeres (arrowheads in C1) and associated with sarcomeric  $\alpha$ -actinin (arrowheads in C2–3). Bar=20  $\mu$ m.



**Fig. 5.** Percentage incidence of the expression of sarcomeric proteins and striation in cultured posterior blastoderm shown in Figures 3 and 4. After 48 hr (h) in culture, explants expressed sarcomeric proteins (sarcomeric  $\alpha$ -actinin, 61%; smooth muscle  $\alpha$ -actin (SMA), 95%; Z-line titin, 56%; sarcomeric myosin, 48%); however, they did not yet show an apparent striation (hatched column). After 72 hr, explants expressed sarcomeric proteins (sarcomeric  $\alpha$ -actinin, 92%; SMA, 100%; Z-line titin, 94%; sarcomeric myosin, 63%). I-Z-I proteins were incorporated into the striation in 75% of explants or more (sarcomeric  $\alpha$ -actinin, 75%; SMA, 81%; Z-line titin, 83%), and sarcomeric myosin in 37% of explants. There was a significant difference in the percentage incidence of expression or striation among four sarcomeric proteins. The results of each individual pairwise comparison were indicated. Note that there was no significant difference in the incidence of expression or striation of I-Z-I proteins and that the incidence of expression or striation of I-Z-I proteins was significantly higher than that of sarcomeric myosin. Asterisk (\*) indicates that there is 5% significant difference ( $P < 0.05/6$ , using Bonferroni's correction). NS, no significant difference; n, number of explants tested; SMA, smooth muscle  $\alpha$ -actin.

sion or striation of I-Z-I proteins was significantly higher than that of sarcomeric myosin.

It has been reported that the posterior blastoderm contains not only prospective heart cells but also skeletal muscle [14]. Therefore, we checked the expression of a skeletal muscle marker, 12/101, in cultured posterior blastoderm. However, there was no detectable immunoreactivity to the anti-skeletal sarcoplasmic reticulum antibody (12/101 antibody, data not shown), indicating that mature skeletal muscle had yet to develop after 72 hr in culture. This may be because mature skeletal muscle does not develop *in ovo* during 72 hr of incubation [1].

#### IV. Discussion

In the present paper, we described that cultured posterior blastoderm (epiblast cells associated with subjacent hypoblast cells) in serum-free defined medium was able to generate mature myofibrils with sarcomeres. At the onset of myofibrillogenesis (after 48 hr in culture), actin filaments of SMA were associated with periodic tiny bead-like deposits of sarcomeric  $\alpha$ -actinin and Z-line titin; at this time, the deposition of sarcomeric myosin appeared to be diffuse. After 72 hr, although 75% or more of explants showed sarcomeric staining of sarcomeric  $\alpha$ -actinin, SMA and Z-line titin, sarcomeric myosin was incorporated into the sarcomere in only 37% of explants. Therefore, in this culture model, myofibrillogenesis took place after 48 hr in culture and mature myofibrils with sarcomeres developed by 72 hr. Our immunohistochemical observation for myofibrillogenesis supported the schema that the nascent I-Z-I components consisting of SMA, sarcomeric  $\alpha$ -actinin, and Z-line titin might be generated independently of sarcomeric myosin-associated A-bands, which would be recruited into the I-Z-I structure to generate mature myofibrils [16, 19, 30]. Therefore, the developmental process of the myofibrillogenesis observed in the cultured posterior blastoderm mimicked that *in vivo* [8], in cultured cardiomyocytes [16, 19] and in cultured anterior lateral plate mesoderm (heart mesoderm) [7, 24, 25].

The earliest development of the heart at gastrula stage has been investigated extensively using avian embryos, as they are easy to handle *in vivo* and *in vitro*. During gastrulation, presumptive heart cells are committed to the cardiac lineage and terminal differentiation occurs at around the gastrula to neurula stages (for reviews [4, 21]). This specification and the subsequent terminal differentiation processes are regulated by endoderm-derived bone morphogenetic protein (BMP)-2/4, fibroblast growth factor (FGF)-8 and antagonists to the canonical Wnt-pathway [2, 9, 20, 22, 24, 27]. In pregastrula embryos, the signaling regulating the formation of the heart-forming mesoderm as well as cardiomyocytes is not yet well understood. Explantation experiments using an avian blastoderm culture showed that the posterior epiblast develops into heart mesoderm as well as cardiomyocytes, if cultured with associated hypoblast or medium supplemented with activin [18, 23, 31]. There-

fore, tissue interaction between the posterior epiblast and hypoblast is required to generate heart mesoderm/cardiomyocytes [23, 31]. However, the molecular mechanisms that regulate the induction of heart mesoderm/cardiomyocytes from the posterior epiblast remain uncertain. To investigate the molecular mechanisms regulating early cardiogenesis, our serum-free culture model would be convenient, as the histogenesis *in vitro* faithfully reflects that *in vivo* and loss-of-function and gain-of-function experiments are easy to perform.

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