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Maternal and zygotic activin signaling promotes adequate pattern and differentiation of mesoderm through regulation of pluripotency genes during zebrafish development

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ABSTRACT To investigate the role of maternal Activin-like factors in the preservation of stemness and mesendoderm induction, their effects were promoted and inhibited using synthetic human Activin A or SB-505124 treatments, respectively, before the maternal to zygotic transition (MZT). To study the role of zygotic Activin-like factors, SB-505124 treatment was also used after the MZT. Promoting the signaling intensity of maternal Activin-like factors led to premature differentiation, loss of stemness, and no mesendoderm malformation, while its alleviation delayed the differentiation and caused various malformations. Inhibition of the zygotic Activin-like factors was associated with suppressing the *ndr1, ndr2, oct4* (*pou5f3*), *mycb* and *notail* transcription as well as differentiation retardation at the oblong stage, and a broad spectrum of anomalies in a dose-dependent manner. Together, promoting the signal intensity of maternal Activin-like factors drove development along with mesendodermal differentiation, while suppression of the maternal or zygotic ones maintained the pluripotent state and delayed differentiation.

KEY WORDS: *ndr1, differentiation, mesendoderm, zebrafish*

Introduction

NODAL, a glycoprotein belonging to the Transforming Growth Factor β(TGFβ) superfamily, is a conserved factor in vertebrates participating in multiple developmental events (Bennett *et al.*, 2007, Jia *et al.*, 2008, Whitman, 2001). Although there is one *Nodal* gene in mammals, due to the whole genome duplication occurred in the evolution of teleost, the zebrafish genome contains three NODAL orthologues, including *ndr1* (s*quint /sqt*), *ndr2* (*cyclops/cyc*), and *ndr3* (s*outhpaw*) (Schier, 2009). NODAL ligand transmits the signal through a tetrameric receptor complex, consisting of two Activin type I and two Activin type II serine/threonine kinase receptors. Following ligand binding, both the type I (Activin receptor Like Kinase 4/5/7 (ALK4/5/7)) and type II receptors (ActRIIB) in association with the EGF-CFC co-receptor (*Crypto* and *oep/tdgf1* in mice and zebrafish, respectively) phosphorylates SMAD2/3 (Hasanpour and Eagderi, 2020, Quail *et al.*, 2013). The phosphorylated SMAD2/3 homo- or heterodimerize and then form a triplex complex by SMAD4, and subsequently translocate into the nucleus, where in association with other transcription factors regulate their target genes expression (Hasanpour and Eagderi, 2020, Wu and Hill, 2009). In addition to NODAL, Activins, Growth Differentiation Factor 1/3 (GDF1/3), and other TGFβ-related ligands (collectively termed Activin-like factors) can also regulate gene expression through the SMAD2/3 signaling pathway (Hagos and Dougan, 2007, Hagos *et al.*, 2007, Shen, 2007, Sun *et al.*, 2006).

Abbreviations used in this paper: A2, Activin 20 ng/ml; A5, Activin 50 ng/ml; ALK4/5/7, Activin receptor Like Kinase 4/5/7; cyc or ndr2, cyclops; DMSO, dimethyl sulfoxide; EGF-CFC, Epidermal Growth Factor-Crypto Frl1 Cryptic co-receptor; EVL, enveloping layer; flh, floating head; hESCs, human embryonic stem cells; hpf, hours post fertilization; MZT, maternal to zygotic transition; ndr3, southpaw; ntl, notail; oep, one eyed pinhead; PBS, phosphate-buffered saline; RE, relative expression; RIN, RNA Integrity Number; SB-A100, SB-505124 100μM; SB-A30, SB-505124 30μM; SB-A50, SB-505124 50μM; SB-B50 : SB-505124 100 μM; SB-B50, SB-505124 50 μM; sqt or ndr1, squint; TGFβ, Transforming Growth Factor β; WT, Wild Type.

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Like the multifunctional morphogens, Activin-like factors may have different roles during development (Chen and Schier, 2001, Hagos and Dougan, 2007, Thisse *et al.*, 2000). In particular, Activin/SMAD signaling is critical for self-renewal and pluripotency of both hESCs and mESCs, (human and mice embryonic stem cells) where it participates in the control of the core transcriptional network characterizing pluripotency, which includes NANOG, OCT4, MYC, etc. (Brown *et al.*, 2011). Moreover, Activin/SMAD cooperates with NANOG and OCT4 to repress the expression of neuroectoderm promoting gene, namely SIP1 (smad interacting protein 1) (Chng *et al.*, 2011, Fathi *et al.*, 2017, Xu *et al.*, 2008). Further investigation revealed that Activin/SMAD and FGF synergize to inhibit BMP signaling, which in turn prevents endoderm formation and sustains the expression of the core pluripotency markers. The cascade also induces FGF2 and WNT3 expression that are essential to sustain stemness (James *et al.*, 2005, Oshimori and Fuchs, 2012). However, no evidence has yet been reported on the role of Activin-like factors in sustaining stemness in zebrafish.

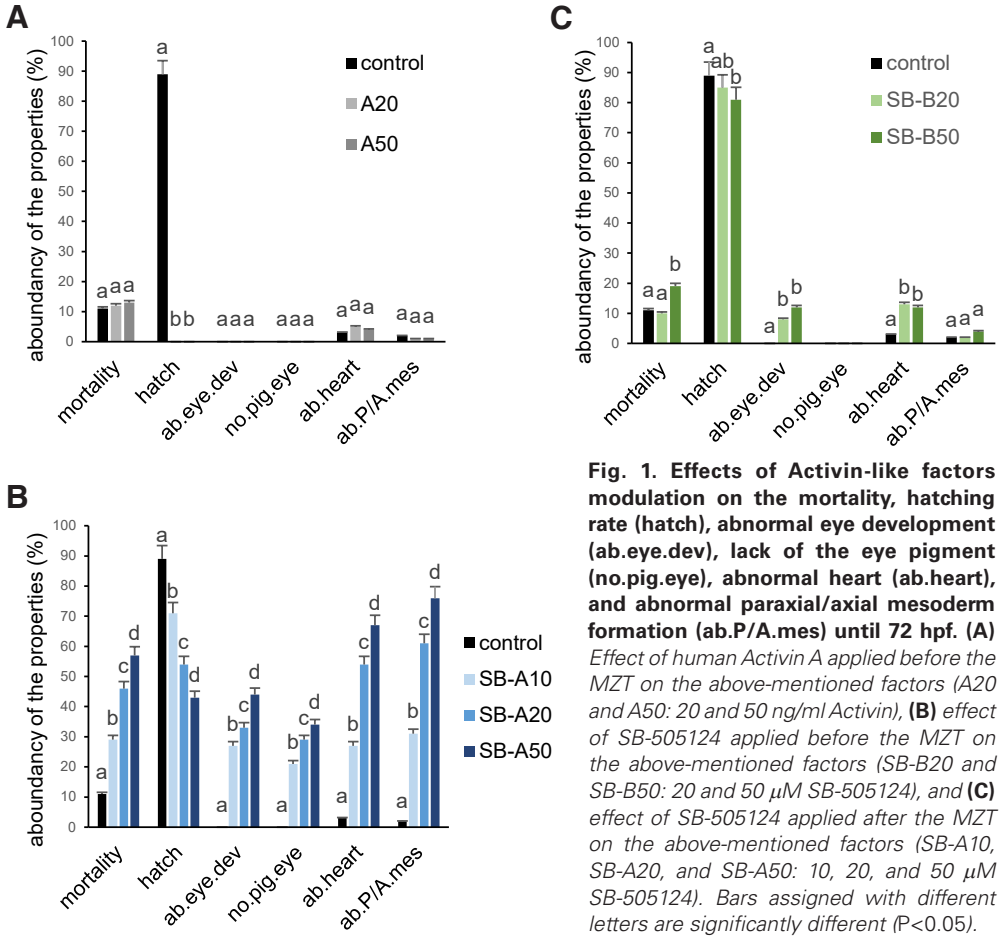

Fig. 1. Effects of Activin-like factors modulation on the mortality, hatching rate (hatch), abnormal eye development (ab.eye.dev), lack of the eye pigment (no.pig.eye), abnormal heart (ab.heart), and abnormal paraxial/axial mesoderm formation (ab.P/A.mes) until 72 hpf. (A) *Effect of human Activin A applied before the MZT on the above-mentioned factors (A20 and A50: 20 and 50 ng/ml Activin),* **(B)** *effect of SB-505124 applied before the MZT on the above-mentioned factors (SB-B20 and SB-B50: 20 and 50 μM SB-505124), and* **(C)** *effect of SB-505124 applied after the MZT on the above-mentioned factors (SB-A10, SB-A20, and SB-A50: 10, 20, and 50 μM SB-505124). Bars assigned with different letters are significantly different (*P<0.05*).*

Despite its crucial role in ESCs pluripotency, Activin/SMAD signaling also functions *in vitro* and *in vivo* to drive the differentiation of pluripotent stem cells toward mesendoderm. Indeed, genetic analysis in vertebrates, e.g. zebrafish, clarifies the core of a conserved transcriptional pathway wherein Activin/Smad is mediated separately by Gata5 (*faust*) and Mixer/Mezzo (*bonnie and clyde*) to activate Sox32 (*casanova*), whose expression is required for terminal differentiation of the endoderm through definitive endoderm marker expression called Sox17 (Gong and Korzh, 2004, Tam *et al.*, 2003). Furthermore, Nodal/Activin activates Fgf signaling in the equatorial region of the zebrafish embryo. Fgf/Erk activity, directly and indirectly, promotes mesoderm formation by eliciting the *notail* (n*tl*, *tbxta*) expression and antagonizing the endoderm formation in the dorsal region (Mizoguchi *et al.*, 2006, Poulain *et al.*, 2006). Notail (*ntl*) is a T-box transcription factor involved in the gene programs required for mesoderm differentiation, whose expression parallels the induction of differentiation processes during the zebrafish development (Amacher *et al.*, 2002, Schulte-Merker *et al.*, 1994), i.e. the sole presence of the pluripotent markers cannot determine the pluripotency state of the embryonic stem cells in zebrafish. Therefore, the onset of the germ layers differentiation is

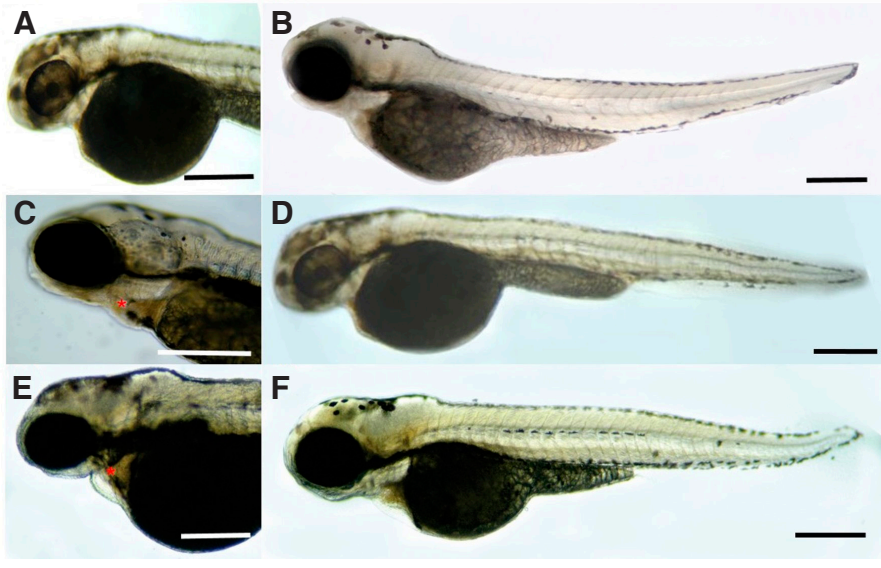

Fig. 2. Effect of human Activin A applied before the MZT on the morphological and physiological characteristics (bar: 800 μm). *The control group at 72 hpf* **(A-B)**, *the A20 group (Activin 20 ng/ml) at 72 hpf* **(C-D)**, *and the A50 group (Activin 50 ng/ml) at 72 hpf* **(E-F)**. *The A20 and A50 groups were normal without any significant differences with the control one* **(D,F)**, *however, the mild pericardial dilation of the heart was observed in some larvae (C,E).*

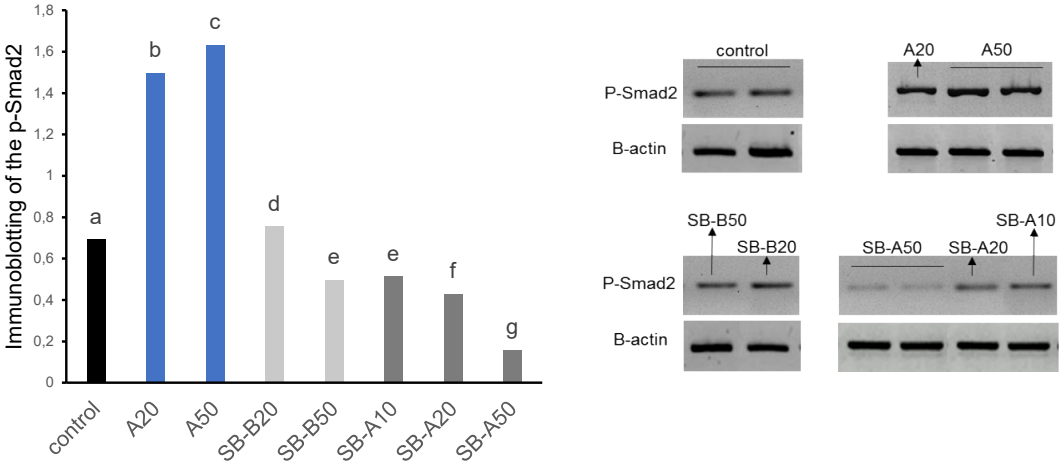

Fig. 3. Immunoblotting analysis of the phosphorylated-Smad2 (p-Smad2) at the oblong stage. *A20 and A50: 20 and 50 ng/ml Activin applied before the MZT; SB-B20 and SB-B50: 20 and 50 μM SB-505124 applied before the MZT; SB-A10, SB-A20 and SB-A50: 10, 20 and 50 μM SB-505124 applied after the MZT. The bars assigned with different letters are significantly different (*P<0.05*).*

a critical point for loss of stemness condition, and *notail* is used as a differentiation index in this regard (Xiao *et al*., 2016). Together, it seems that Activin-like factors have a bi-functional role in pluripotency maintenance and differentiation induction.

In addition, the preliminary stages of embryonic development occur in the absence of de novo transcription and rely on maternal mRNAs and proteins deposited in the egg during oogenesis (Giraldez *et al*., 2006). Following the transcriptional quiescence, the zygotic transcription starts, and maternal control of development declines (Giraldez *et al*., 2006). This period, known as the maternal-to-zygotic transition (MZT), occurs at the 1K-cell stage (Giraldez *et al*., 2006, Kane and Kimmel, 1993). The roles of maternal and zygotic Activin-like factors during zebrafish development are highly controversial (Chen and Schier, 2001, Hagos and Dougan, 2007, Thisse *et al*., 2000). Several experiments were conducted to define their exact roles in zebrafish. However, the findings are controversial (Hagos *et al*., 2007, Sun *et al*., 2006). Other experiments were performed by injecting antisense morpholino oligonucleotides, which have many pivotal disadvantages, including incomplete knockdown and occasional off-target non-specific deleterious effects, and their efficacy diminishes following developmental progression.

To investigate the roles of maternal and zygotic Activin-like factors in zebrafish development, a pharmacologic experiment was performed using Activin as an activator and SB-505124 as an inhibitor of the pathway. Activin binds to Activin type II receptors and initiates a cascade reaction leading to Smad2/3 phosphorylation. In contrast, SB-505124, the inhibitor of Activin type I receptors, prevents activation of the pathway (Laping *et al*., 2002). The effects of maternal Activin-like factors were studied by treating the zebrafish embryos with Activin and SB-505124 before the 1K-cell stage. In contrast, the role of zygotic Activin-like factors was assessed by SB-505124 treatments applied from the 1K-cell stage. In this regard, Activin was used at 20 (A20) and 50 (A50) ng/ml, and SB-505124 at 20 and 50 μM (SB-B20 and SB-B50, respectively) before the 1K-cell stage. Following the MZT, SB-505124 was also applied at 10, 20, and 50 μM (SB-A10, SB-A20, and SB-A50, respectively). The effects of treatments were extensively studied based on morphological and physiological features of the embryos and measuring the *ndr1, ndr2, mycb, oct4* (p*ou5f3*) and *notail* (n*tl*) mRNA levels at the 1K-cell (3 hours post-fertilization (hpf)), oblong (3.65 hpf), dome (4.33 hpf) and shield (6 hpf) stages using the RT-qPCR.

Results

Promoting the maternal Activin-like factors through Activin exposure

The mortality rate showed no difference in Activin-treated groups and the control (Fig. 1A). Morphological assessments of eye development and its pigmentation, heart, axial, and paraxial mesoderm formation in the A20 and A50 groups were normal, without any significant differences with those of the control (*P*>0.05, Figs. 1, 2).

Immunoblotting analysis of the phosphorylated-Smad2 at the oblong stage demonstrated that Activin treatments effectively promote the Smad2/3 pathway (Fig. 3). Activin treatments promoted *ndr1* expression in a dose-dependent fashion at the 1K-cell stage, reduced its mRNA level at the oblong stage at the higher dose (50 ng/ml), and had no effects at the dome and shield stages (Fig. 4A). The *ndr2* was first transcribed at the oblong stage, and then its levels went up until the shield stage. Aside from the dome stage, the effect of activin treatments on *ndr2* expression was insignificant (Fig. 4B).

Maternal transcripts of the *oct4* (*pou5f3*), which is involved in maintaining pluripotency, increased at the 1K-cell stage due to the addition of its zygotic versions and then surged at the oblong and dome stages. Administration of Activin was associated with a significant plunge in Oct4 (*pou5f3*) mRNA level, continuing until the shield stage when no difference was seen among the groups (Fig. 4C). The Mycb, a pleiotropic transcription factor participating in various developmental programs and establishment of pluripotency, followed the same patterns seen in the *oct4* (*pou5f3*). The only difference was that the decreasing effects of Activin treatments on Mycb mRNA levels had been restricted until the dome stage (Fig. 4D).

The maternal transcripts of *notail* were not detected at the 256-cell stage. Lack of the *notail* transcripts continued until the oblong stage, when it increased strongly until the shield stage in the control group. Activin treatments exerted increasing effects on the *notail* zygotic expression at the oblong and dome stages, but no significant difference was found between the groups at the shield stage (Fig. 4E). Furthermore, morphological landmarks ascribed to the oblong and dome stages appeared in advance in activin-treated groups (about 10-15 min).

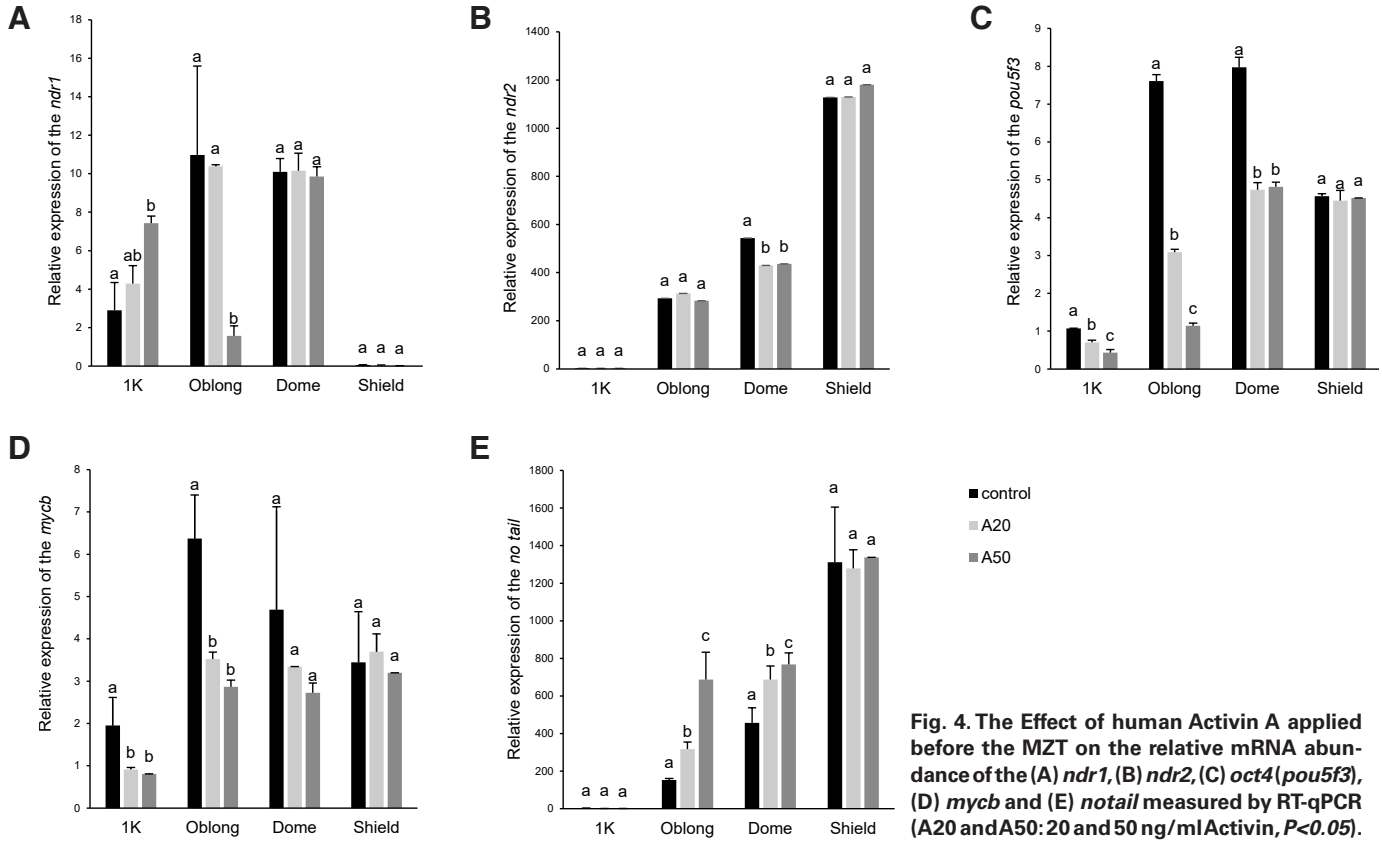

Fig. 4. The Effect of human Activin A applied before the MZT on the relative mRNA abundance of the (A) *ndr1*, (B) *ndr2*, (C) *oct4* (*pou5f3*), (D) *mycb* and (E) *notail* measured by RT-qPCR (A20 and A50: 20 and 50 ng/ml Activin, *P<0.05*).

Inhibiting the maternal Activin-like factors through SB-505124 exposure before the MZT

A significant increase in the mortality rate (19%, 57/300) was observed in the SB-B50 treatment compared to the control group ($P>0.05$). Developmental procedures of the eye, heart, notochord, and somites in the SB-B20 and SB-B50 groups were similar to those of the control. However, the prevalence of cardiologic disorders, including functional and anatomical defects, e.g. abnormal dilation of the pericardium in the front of the yolk, was significantly higher. In addition, 8% (24/300) of the SB-B20 embryos and 12% (36/300) of the SB-B50 revealed abnormal eye development, ranging from their convergence to synophthalmia (cyclopia) ($P<0.05$, Fig. 1B and Fig. 5).

Immunoblotting analysis of the phosphorylated-Smad2 at the oblong stage showed that SB-505124 treatments before the MZT effectively inhibit the Smad2/3 pathway (Fig. 3). SB-505124 treatments before the MZT reduced the *ndr1* mRNA levels at the 1K-cell and oblong stages. However, it was associated with the *ndr1* mRNA abundancy at the dome and shield stages (Fig. 6A). The effect of maternal Activin/Smad suppression on *ndr2* mRNA levels was first detected at the oblong stage, when they were lower than the control group; then, they soared until the shield stage ($P<0.05$, Fig. 6B).

SB-505124 usage before the MZT led to a significant increase in the *oct4* (*pou5f3*) mRNA levels at the 1K-cell and oblong stages. No effects were registered for the next stages (Fig. 6C). The *mycb* followed the *oct4* (*pou5f3*) pattern except at the 1K-cell stage, when it showed no difference among the groups (Fig. 6D).

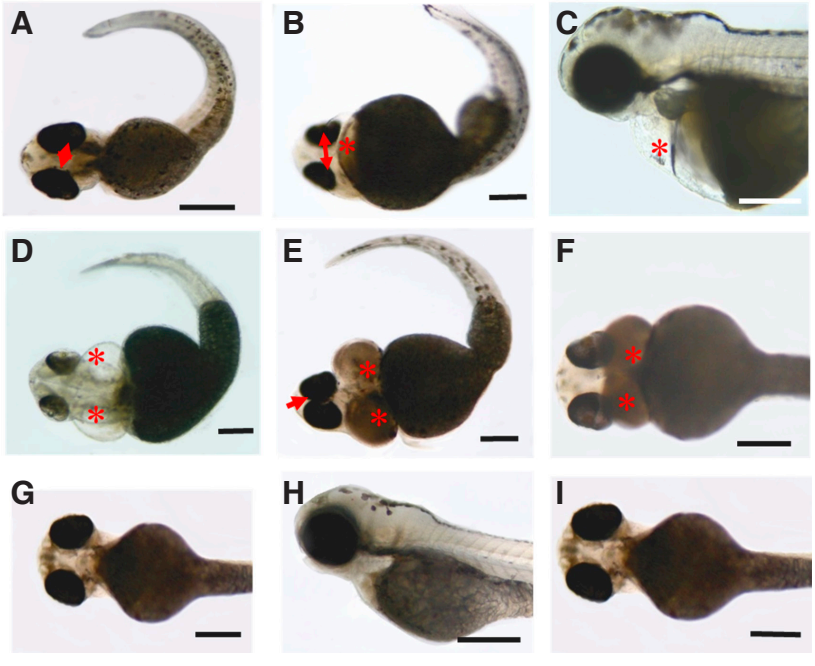

Fig. 5. The effect of SB-505124 applied before the MZT on the morphological and physiological characteristics (bar: 800 μm). *The SB-B20 group (20 μM SB-505124) at 48 hpf* **(A-B)** *and at 72 hpf* **(C)**, *the SB-B50 group (50 μM SB-505124) at 48 hpf* **(D-E)** *and at 72 hpf* **(F)**, *and the control group at 48 hpf* **(G)** *and at 72 hpf* **(H-I)**. *Abnormal dilation of the pericardium (B-F) and the eye convergence (A-B and E) have been indicated.*

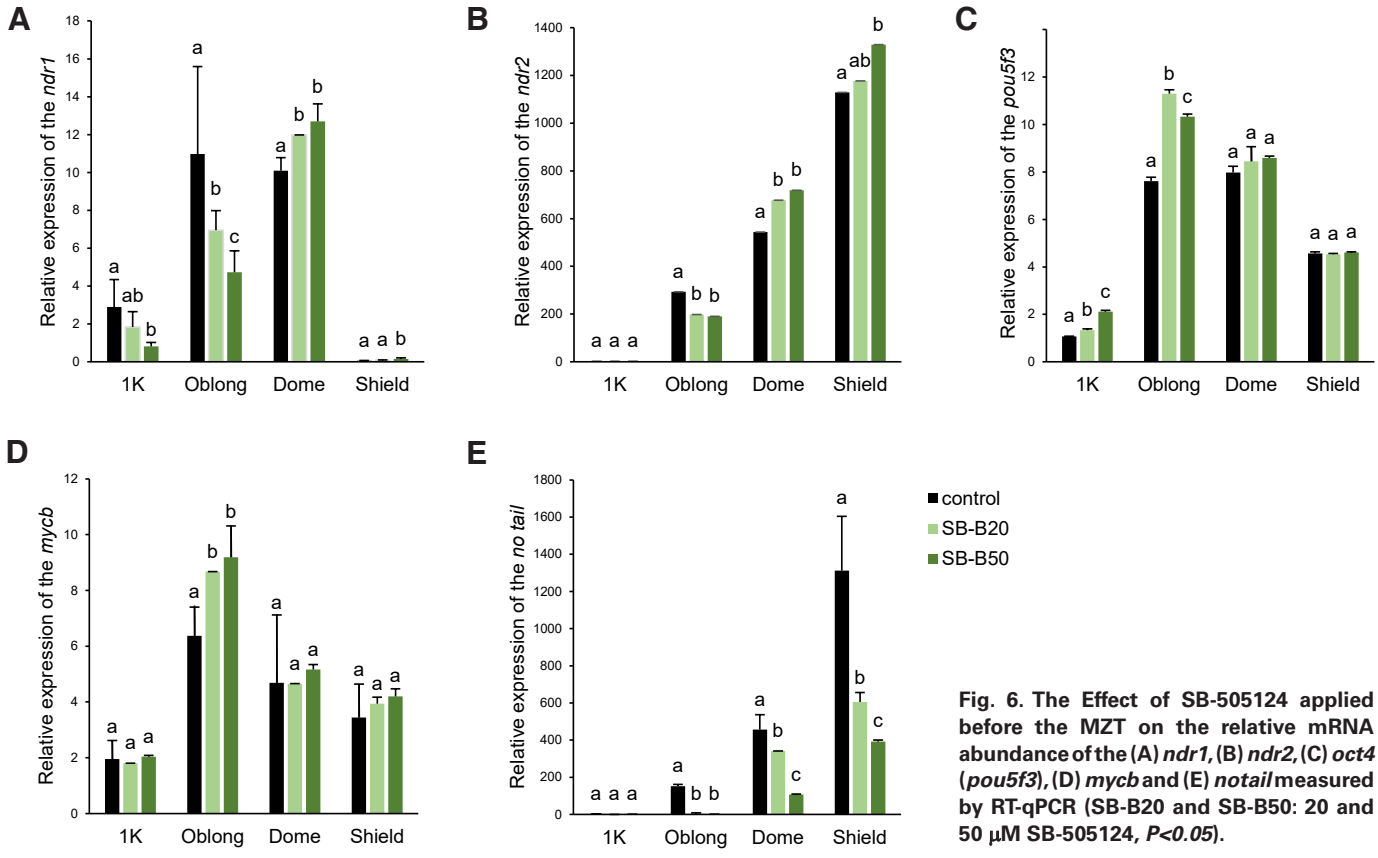

Fig. 6. The Effect of SB-505124 applied before the MZT on the relative mRNA abundance of the (A) *ndr1*, (B) *ndr2*, (C) *oct4* (*pou5f3*), (D) *mycb* and (E) *notail* measured by RT-qPCR (SB-B20 and SB-B50: 20 and 50 μM SB-505124, *P<0.05*).

Application of SB-505124 before the MZT markedly reduced *notail* expression ($P<0.05$) at the oblong stage, as it was not detected. Globally, SB-B20 and SB-B50 treatments decreased *notail* expression at the dome and shield stages ($P<0.05$, Fig. 6E). Besides, the morphological landmarks ascribed to the oblong and dome stages appeared about 10-15 minutes later, i.e. inhibition of the Activin-like factors delays the induction of differentiation.

Inhibiting the zygotic Activin-like factors through SB-505124 exposure after the MZT

The mortality rate of the SB-505124 treated embryos after the MZT rocketed in a dose-dependent manner ($P<0.05$). The hatching rate considerably declined from 89% (267/300) in the control group to 71% (213/300) in the SB-A10, 54% (162/300) in the SB-A20, and 43% (129/300) in the SB-A50 ($P<0.05$, Fig. 1C*)*. Axial and paraxial mesoderm malformations, including the notochord truncation and fused somites or lack of them except in the tail region, were observed in 31% (93/300) of the SB-A10 embryos, significantly higher than the control group ($P<0.05$, Fig. 7). This acute shortage of the mesendodermal derivatives in the SB-A20 and SB-A50 groups markedly increased by 61% and 76% (183/300 and 228/300) one by one (Fig. 1C*)*. Anatomical and physiological evaluations indicated heart dilation with ceasing of the heartbeat and blood circulation in about one-quarter of the SB-A10 treated embryos and more than half of the other treated groups (Fig. 8). The ectoderm and its derivatives developed in contrast with mesendodermal defects. However, 27% (81/300) of the SB-A10, 33% (99/300) of the SB-A20, and 44% (132/300) of the SB-A50 group displayed synophthalmia.

Immunoblotting analysis of the phosphorylated-Smad2 at the oblong stage showed that SB-505124 treatments after MZT effectively inhibit the Smad2/3 pathway (Fig. 3). SB-505124 exposure after MZT significantly reduced *ndr1*, *ndr2*, *oct4* (*pou5f3*), and *mycb* expression at the oblong, dome, and shield stages (Fig. 9 A-D). Application of SB-505124 after the MZT suppressed *notail* expression at the oblong stage, thereby delaying differentiation. Inhibition of the Activin type I receptors after the MZT was associated with a dose-dependent decrease in *notail* expression at the dome and shield stages (Fig. 9E). Besides, detecting the morphological landmarks ascribed to each stage of development was associated with a significant delay in these treatments.

Discussion

The roles of maternal and zygotic Activin-like proteins are highly controversial (Chen and Schier, 2001, Hagos and Dougan, 2007, Thisse *et al.*, 2000). Therefore, we investigated their effects on the morphological and physiological parameters as well as the expression of some key genes. Although morphological assessment of the A20 and A50-embryos confirmed their normal appearance, the prevalence of the disorders in the SB-B20 and SB-B50 groups was significantly higher and confirmed the role of maternal Activin-like factors during the development. In line with our results, it has been reported that maternal *ndr1* is necessary to form a part of dorsal and anterior tissues in some genetic backgrounds (Hagos and Dougan, 2007, Hagos *et al.*, 2007). Other works showed that maternal Activin-like factors are sufficient for mesoderm expansion and axis formation, and there is no need for zygotic Activin (Hyodo

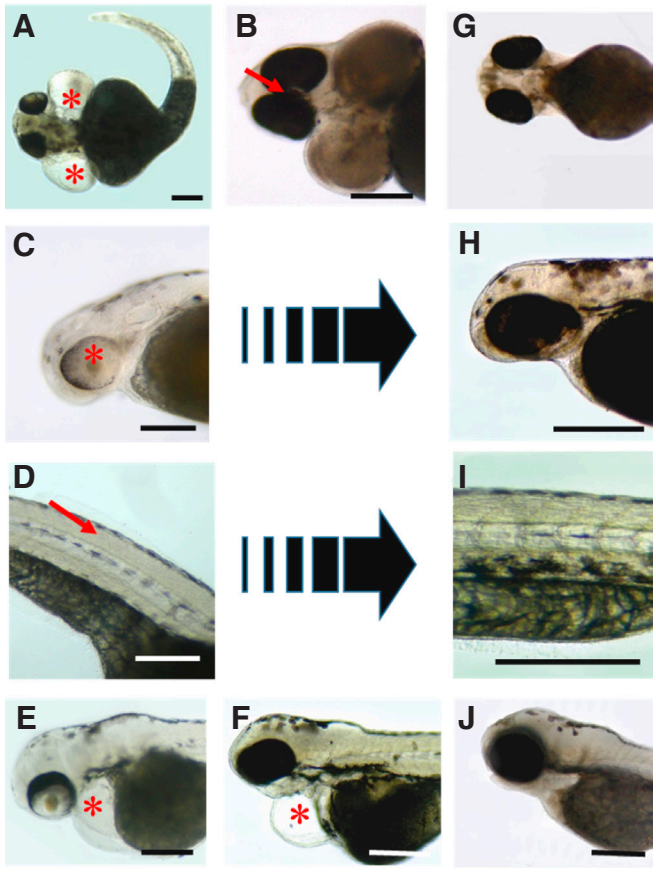

Fig. 7. The effect of SB-505124 applied before the MZT on the morphological and physiological characteristics (bar: 800μm). *The SB-A10 group (10 μM SB-505124) at 48 hpf* **(A-D)** *and at 72 hpf* **(E-F)**, *the control group at 48 hpf* **(G-I)** *and at 72 hpf* **(J)**. *The notochord truncation and fused somites (A and D respectively), heart dilation (A-B and E-F), endodermal defects (E-F), eye synophthalmia, and lack of its pigmentation (B-C and E) have been indicated.*

et al., 2004, Thisse *et al.*, 2000). It seems that the effectiveness of Activin treatments in influencing morphological indices did not equal SB-505124 treatments and might be affected by the additional complexities of tissue thickness; and higher application of Activin or its mRNA injection can probably create the reverse effects seen in the next group. Together, our findings substantiated the role of maternal Activin-like factors, at least in some genetic backgrounds.

Inhibition of the zygotic Activin-like factors by SB-505124 after the MZT led to a broad spectrum of malformations in mesendoderm derivatives, dose-dependently. Principally, the *ndr1* and *ndr2* redundantly pattern the germ layers in a dose-dependent manner. In this regard, during the blastula stage, the Activin/Smad signaling pathway directly regulates transcription of the *bonni and clyde* (Mixer and Mezzo) and *faust* (Gata5), which provoke the *casanova* (Sox32) expression. Sox32 acts as an upstream regulator of the Sox17, considered a definitive endoderm marker (Gong and Korzh, 2004, Tam *et al.*, 2003). In addition, Activin-like factors indirectly, through Fgf/Erk activation, promote the *notail* expression known as a pan-mesodermal marker (Mizoguchi *et al.*, 2006, Poulain *et al.*, 2006). Therefore, our finding was in line with earlier findings.

Activin administration brought about an increase in *ndr1* and *notail* mRNA levels, consistently suppressing either maternal or

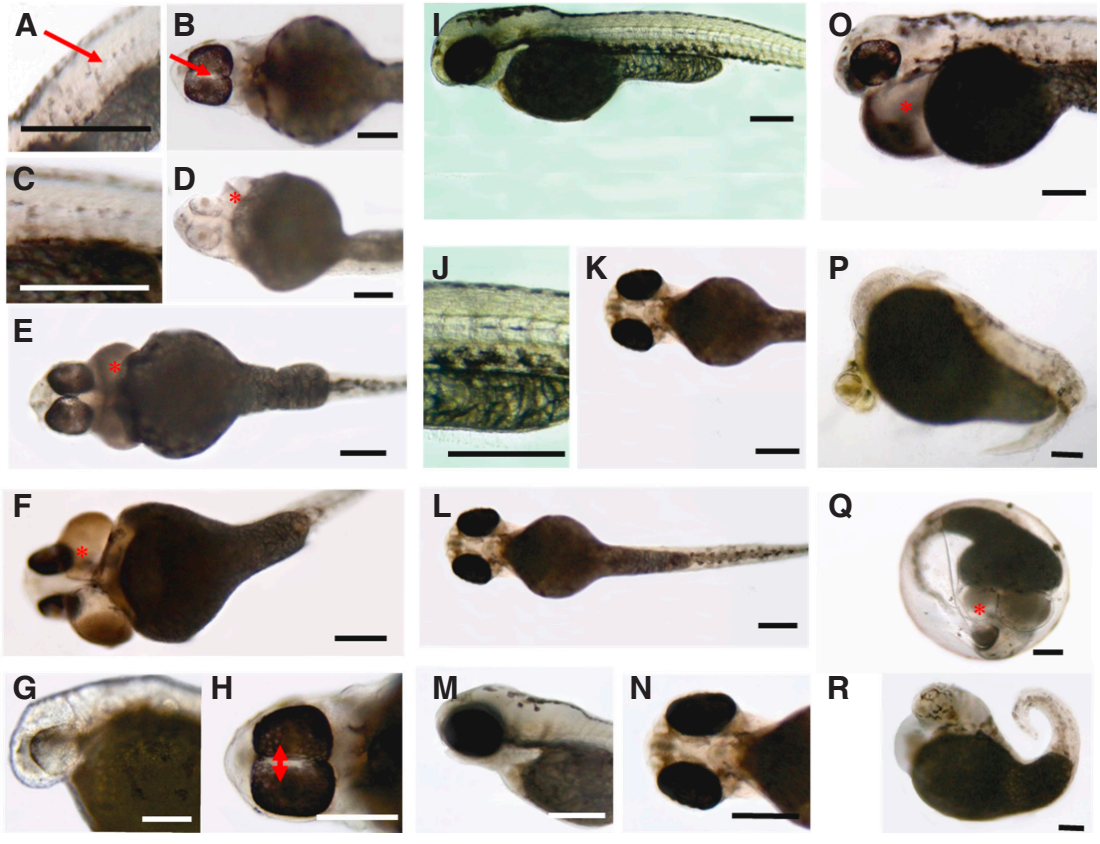

Fig. 8. The effect of SB-505124 applied after MZT on the morphological and physiological characteristics (bar: 800μm). *The SB-A20 group (20 μM SB-505124) at 48 hpf* **(A-E)** *and at 72 hpf* **(F-H)**, *the control group at 48 hpf* **(I-K)** *and at 72 hpf* **(L-N)**, *and the SB-A50 group (50 μM SB-505124) at 48 hpf* **(O-P)** *and at 72 hpf* **(Q-R)**. *The notochord truncation and fused somites (A, C and P, Q respectively), heart dilation (D-F, and O-R), endodermal defects (F-G, and O-R), eye synophthalmia, and lack of its pigmentation (B, D, and G) have been indicated.*

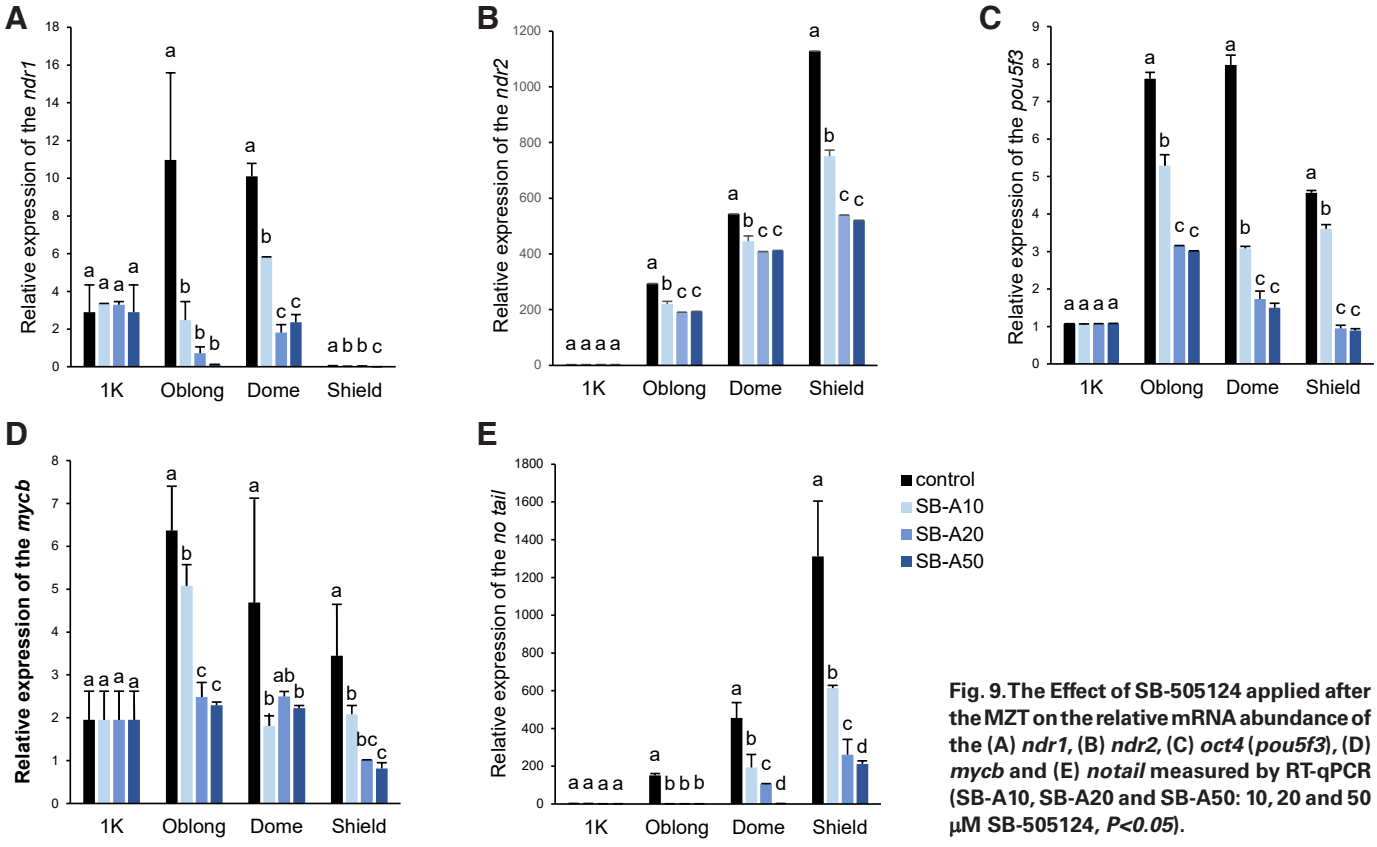

Fig. 9.The Effect of SB-505124 applied after the MZT on the relative mRNA abundance of the (A) *ndr1,* (B) *ndr2,* (C) *oct4* (*pou5f3*), (D) *mycb* and (E) *notail* measured by RT-qPCR (SB-A10, SB-A20 and SB-A50: 10, 20 and 50 μM SB-505124, *P<0.05*).

zygotic Activin/Smad transduction pathway lowered transcription of the two genes. In line with our findings, Yang *et al.,* (2017) reported that Activin treatment promotes *ndr1* expression due to higher activation of the cellular signaling pathway (Yang *et al.*, 2017). However, reduced *ndr1* expression at the oblong stage in our Activin treated groups was similar to the observation in *Xenopus laevis* in which FoxH1, the direct target of the Nodal, in association with Grg4 (groucho related gene 4) negatively regulates Nodal gene expression to ensure proper formation of the primary germ layers (Osada *et al.*, 2000, Reid *et al.*, 2016). The aforementioned results with regard to the modulation effects of Activin-like factors on *ndr1* and *notail* were consistent with the necessity of *ndr1* signaling for mesoderm formation, as *notail* expression fails to initiate in embryos with diminished *ndr1* signaling (Dougan *et al.*, 2003, Schulte-Merker *et al.*, 1994). Another study revealed that *notail* is downregulated in the dorsal side of the *ndr1* mutant embryos because Ndr1 indirectly promotes *notail* encoding through Fgf/Erk activation (Mizoguchi *et al.*, 2006, Poulain *et al.*, 2006). Together, *ndr1* increase due to Activin treatments was responsible for earlier activation of the *notail* gene; i.e.it brought forward the starting point of *notail* encoding. Conversely, *ndr1* decrease because of SB-505124 application delayed and decreased *notail* expression.

In the control group, *notail* expression initiated at the oblong stage, when its level went up stage-by-stage in accordance with notochord development (Garnett *et al.*, 2009, Mullins, 1998, Schulte-Merker *et al.*, 1994). Promoting the signaling intensity of maternal Activin-like factors was associated with a slash in *oct4* (p*ou5f3*) and *mycb* mRNA levels. At the same time, the aforementioned treatment led to earlier activation of the *notail* and an increase in *notail* and *ndr1* expression. Moreover, inhibiting the maternal Activin-like factors promoted the expression level of the *oct4* (*pou5f3*) and *mycb*, delayed *notail* encoding, and downregulated *notail* and *ndr1* transcription. *Oct4* (*pou5f3*) participates in genome-wide chromatin reprogramming to achieve transient totipotency and zygotic genome activation (Lee *et al.*, 2013, Miao *et al.*, 2020). In all organisms, *oct4* (*pou5f3*) expression is associated with the cells at the pluripotency stages and during loss of pluripotency (Onichtchouk, 2012), although this master pluripotency marker is downregulated upon differentiation (Perrett, 2008). Therefore its presence and lack of primary differentiation markers, especially the *notail,* demonstrates the cells' stemness state (Xiao *et al.*, 2016). As Gao *et al.,* (2020) reported, *oct4* (*pou5f3*) ablation leads to a burst of premature expression of the genes which normally regulate tissue differentiation at organogenesis stage (Gao *et al.*, 2020). In another study, abrogation of the *oct4* (*pou5f3*) expression in mESCs, led to their differentiation along the trophoblast lineage (Kotkamp *et al.*, 2014, Radzisheuskaya and Silva, 2014). Taken together, Activin usage leading to a significant decrease in *oct4* (*pou5f3*) and *mycb* expression, along with *ndr1* and consequently notail transcription enhancement, elicited premature differentiation. While SB-505124 administration, by improving the *oct4* (*pou5f3*) and *mycb* expression as well as decreasing *ndr1* and suppressing *notail* transcription, delayed the differentiation program.

Application of Activin and SB-505124 before the MZT led to a decrease and an increase in *oct4* (*pou5f3*) expression, respectively. However, SB-505124 usage after the MZT caused a decrease in its transcription. Comparison of the results obtained from modulation of the maternal Activin/Smad was consistent with a previous study, confirming that SB-431542, a TGFβ signaling inhibitor, can activate the expression of endogenous *oct4* (*pou5f3*) during reprogramming.

According to this study, SB-431542 can sustain the pluripotency of iPSCs and ESCs by reducing ERK phosphorylation, which is a downstream effector of FGF signaling (Tan *et al*., 2015). Indeed, due to Erk's low binding affinity with the *notail* promoter region, its basal level does not transcribe the *notail* (stemness state). Therefore, following Activin usage, prompt enhancement of the *ndr1* significantly increases the Erk level, which elicits *notail* expression (Itoh *et al*., 2014, Oshimori and Fuchs, 2012, Perrett, 2008). The effects of zygotic Activin/Smad modulation on *oct4* (*pou5f3*) in this study were in line with the fact that Activin/Smad transcribes the key pluripotency genes, which in turn coordinate with Smad2/3 to sustain the stemness of hESCs. Together, Smad2/3 presence is a necessary condition but not sufficient for *oct4* (*pou5f3*) transcription.

Based on our results, *mycb* and *oct4* (*pou5f3*) genes had the same response to treatments. In line with these results, the *myc* gene, which inhibits cell differentiation due to its capacity for epigenetic modifications (Marandel *et al*., 2012, Mitra *et al*., 2019), is regulated by *oct4* (*pou5f3*) during early embryogenesis. This regulatory mechanism is considered to be an evolutionarily conserved feature in vertebrate development (Kotkamp *et al*., 2014), as the transcription levels of the two zebrafish *myc* genes, *mycl1b* and *mych* were reduced in the maternal and zygotic *oct4* (*pou5f3*) mutant embryos (Kotkamp *et al*., 2014).

The *ndr2* expression pattern was similar to that observed in earlier studies (Liang and Rubinstein, 2003, Rebagliati *et al*., 1998). Fluctuations of *ndr2* due to administration of different treatments were in line with the *ndr1*, which is necessary for *ndr2* to achieve the normal level (Dougan *et al*., 2003).

Together, this study revealed a novel function of the maternal and zygotic Activin-like factors in sustaining the stemness and differentiation retardation in the zebrafish embryo.

Materials and methods

Fish maintenance

Adult zebrafish were reared at 28°C with a 14h light/10h dark photoperiod (in the Ontogeny and Biosystematics lab, the University of Tehran). Following natural spawning, Wild Type (WT) embryos were collected and staged according to Kimmel *et al.,* (Kimmel *et al*., 1995). All procedures involving animals were according to the ethical standards of the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

Activin treatments

Due to the sequence conservation of Activin β_A and β_B subunits, recombinant human Activin A can substitute Activin-like factors in other vertebrates (Hyodo *et al*., 2004, Melamed and Sherwood, 2005, Tada *et al*., 1998). Hence, to promote the effects of the maternal Activin-like factors, synthetic human Activin A (Peprotech, Rocky Hill, USA) was applied before the MZT, following the chorion removal. For dechorionation with two pairs of sharply pointed forceps, the embryos were first incubated in 2 mg/ml pronase (Roche, Mannheim, Germany) for 1 min, and rinsed 3-4 times with 8X water (12 ml stock salts per liter dH_2O (stock salts: 40 gr instant ocean sea salt per liter dH_2O)) (Apelqvist *et al*., 1997, Grove and Monuki, 2013, Henn and Braunbeck, 2011, Ho *et al*., 2006, Westerfield, 1995, Westerfield, 2000). After dechorionation, undamaged embryos were transferred to 2% agarose coated plates and treated with Activin A. Activin A was applied on the 1- to 4-cell stage embryos at 20 and 50 ng/ml media, hereafter known as the A20 and A50 groups, respectively (Gurdon *et al*., 1995, Ninomiya *et al*., 1999). Human recombinant activin A was dissolved in the embryo medium (Westerfield, 2000) containing 0.1% bovine serum albumin at a concentration of 20 and 50 ng/ml. Each treatment was performed 15 times with 100 embryos.

SB-505124 treatments

SB-505124, a specific inhibitor of Alk4/5/7, can pass through the chorion, and therefore it was applied on the chorionated embryos. SB-505124 was purchased from Tocris R and D system (Bristol, UK) and stored as a 100 mM stock in DMSO at -20°C. To reduce the maternal Activin-like factors effects, SB-505124 was used before the MZT, on the 1- to 4-cell stage embryos at 20 and 50 μM, which are called the SB-B20 and SB-B50 groups, respectively. To relieve the effects of SB-505124 and recover the receptors at the 1K-cell stage, the embryos were washed out with egg water five times (Hagos and Dougan, 2007, Hagos *et al*., 2007, Jia *et al*., 2008, Sun *et al*., 2006). To examine the effects of the zygotic Activin-like factors, 1K-cell stage embryos were incubated with 10, 20, and 50 μM SB-505124. Hereafter, they are referred to as SB-A10, SB-A20, and SB-A50, respectively. The control embryos were treated with an equivalent concentration of DMSO (50 μM). Each treatment was performed 15 times with 100 embryos.

Morphological assessments

Three replicates per treatment were evaluated morphologically and physiologically until 72 hpf. The embryos were anesthetized using tricaine solution (0.168 mg/ml, IACUC approved) and fixed in 10% neutral buffered formalin for 1 hour at 24°C. They were then kept in 70% ethanol at -4°C. The embryos were photographed in 50% phosphate-buffered saline (PBS) in a glycerol bath.

RNA extraction and RT-qPCR

Total RNA was extracted from the embryos at the 256-cell, 1K-cell, oblong, dome, and shield stages using the RNeasy Mini Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. Its quality was then assessed using the Nanodrop (2000 Thermo-scientific), and only samples with high RNA Integrity Number (RIN) (A2060/A2080 ratios of 1.8-2) were selected. cDNAs were synthesized using Superscript II reverse transcriptase (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. For each sample, 1 μg of total RNA was used to perform the reverse transcription experiments (reaction volume: 20 μl). The real-time qPCR [ABI step one Real-Time PCR and StepOne software V2.3] was performed using 2X SYBR Green PCR Master Mix (Ampliqon, Odense, Denmark) according to the manufacturer's protocol. The real-time PCR conditions were as follows: 5 min at 95°C, [15 sec at 95°C, 10 sec at 58.5°C, 10 sec at 72°C] 35 cycles, 10 sec at 95°C, 1 min at 65°C, and 15 sec at 93°C. The annealing temperature of the *ndr2*, *oct4* (*pou5f3*) and *mycb* was 60°C. The *ndr1* (accession number: NC-007132.7), *ndr2* (accession number: NM_139133.1), *oct4 (pou5f3,* accession number: NM_131112.1), *mycb* (accession number: NM_200172.1), *notail* (accession number: NC-007130.7), and *gapdh* (accession number: NC-007127.7) genes were amplified using these primers:

ndr1-F: (GCAGTTGTCCCACACCAGTA)
ndr1-R: (CTGGCAGGAGGAAAACGGAA)
ndr2-F: (AGATGAACCAGACGCGCATA)
ndr2-R: (CTGACGTTCCGACAAAACCG)
oct4 (pou5f3)-F: (AGCGTCTAGCTTTGCCCTTT)
oct4 (pou5f3)-R: (GCATGTATAAGGCAGGGGCT)
mycb-F: (ATACTCCGCCAAACAGTGGG)
mycb-R: (CGCGTCAGACTTTTTCACCG)
notail-F: (ATCATCTCCTTAGCGCCGTG)
notail-R: (AGCACGGGAAACATTCGTCT)
gapdh-F: (TGTTCCAGTACGACTCCACC)
gapdh-R: (ACCTGCATCACCCCACTTAA)

The relative expression was quantified from the threshold cycle for amplification and normalized to the *gapdh* level (ΔCt). The ΔΔCt and relative expression (RE) of the genes were calculated based on their maternal mRNA levels at the 256-cell stage.

Immunoblotting of the phosphorylated-Smad2

The effectiveness of Activin and SB-505124 treatments was confirmed according to the phosphorylated-Smad2 (p-Smad2) quantification through western blot analysis at the oblong stage. After dechorionation, the oblong-embryos were homogenized in 150 μl SDS buffer and microfuged for 1-2 minutes. The supernatant was then run on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to PVDF blotting paper (Westerfield, 2000). Immunoblotting was conducted using phospho-Smad2 (ser465/467) antibody (#3101, Cell signaling technology) and fluorescein goat anti-rabbit IgG secondary antibody (611-1202; Rockland antibodies and assays). β-Actin was used as a loading control (anti β-Actin antibody N-terminal (ab209869, Abcam)). The results were quantified through the Image-J software (Version 1.46r), with β -actin for normalization.

Statistics

Significant differences at 5% in terms of the survival, hatching rate, morphological characteristics, Smad2 phosphorylation, and relative expression of the genes were analyzed using a one-way analysis of variance (ANOVA) in SPSS software (Version 20). All the graphs were drawn in Microsoft excel 2016. The data represent the mean of three independent experiments, and the error bars indicate the standard deviation.

Declarations

Conflict of interests: The authors declare no conflicts of interest. Availability of data and materials: All data generated or analyzed during this study are included in this published article. Funding: The University of Tehran provided financial support by supplying all the required materials and instruments during the experiment. Authors' contributions: All authors have read and approved the manuscript. Conceptualization, S.H and S.E; Methodology and Investigation, S.H; Software, S.H; Validation and Formal Analysis, S.H and H.P; Resources and Data Curation, M.H; Writing – Original Draft Preparation, S.H and M.H; Writing–Review and Editing, S.H and H.P; Visualization and Supervision, S.E and H.P; Project Administration, S.E; Funding Acquisition, M.H and S.E; Final Approval and Accountability: S.H. and S.E.

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