Mesoderm Signaling Pathways of Early Development in the Zebrafish *Danio rerio*

Ilham J.J. Alshami* and Hussein A. Saud
Department of Fisheries and Marine Resources, College of Agriculture,
University of Basrah, Iraq
*Corresponding Author: ilham.jalil@uobasrah.edu.iq

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ABSTRACT

The zebrafish (*Danio rerio*) is an ideal fish model for molecular and genetic studies. It is relatively easy to keep in the aquarium and to obtain embryos. The ability to follow embryonic development using microscopic and molecular techniques facilitates the study of mesoderm development. Hence, this study was conducted to investigate the role of four transcription factors that include: bone morphogenetic protein (BMP), nodal, fibroblast growth factor (FGF) and sonic hedgehog (SHH) in mesoderm induction, using specific inhibitors (DM, SB431542, SU5402) and Cyclopamine, respectively. Each inhibitor perturbed mesoderm development and in situ hybridisation, using *myl10* specific marker for muscles. Results showed noticeable reduction in gene expression with each treatment, indicating a crucial role for all four transcription factors in mesoderm development.

INTRODUCTION

The mesoderm is one of the three primary germ layers as it forms the muscles during early developing stages. Somites of vertebrates are generated from the paraxial mesoderm **(Wang *et al*.,2019)**. Muscle cell formation is connected to the expression of a complex set of organizer molecules that arise from one or two major groups of transcription factors **(Black & Olson, 1998; Chin *et al*., 1998; Allen *et al*., 2001; Schiaffino *et al*., 2007; Rana *et al*., 2008; Gundersen, 2011)**. In addition, myogenic transcription factors consist of the MEF2 and MyoD groups **(Weintraub *et al*., 1991; Black & Olson, 1998; Wu *et al*., 2000; Bassel Duby & Olson, 2006).**

It is worthy to mention that the myogenic regulatory factors (MRFs) are a set of basic helix –loop-helix (bHLH) transcription factors, consisting of myogenin, MRF4, MyoD and MYF5, that have been shown to play a crucial role in the formation of muscle cells **(Buckingham, 1992; Arnold & Brann, 1996; Pownall *et al*., 2002).**

During embryonic development in zebrafish, *myoD* is initially detected on each side of embryonic shield at 70%-75% epiboly (Weinberg *et al*., 1996). At 80% epiboly, *myf5*

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is expressed in two stripes near the notochord **(Coutelle *et al*., 2001)**. Subsequently, *myogenin* is expressed during segmentaton period **(Weinberg *et al*., 1996).** Remarkably, upstream transcription factors possess an important role in muscle induction, among which PAX7 was reported to play a n essential has role in the induction of *myoD* and *myf5* expression in zebrafish **(Ochi & Westerfield, 2007). Fisher *et al*. (2002)** reported the role of FGF signaling in the activation of *myoD*. T-box transcription factors. Moreover, Ntl and Spt also act as key regulators of *myoD* expression in zebrafish **(Weinberg *et al*., 1996)**.

Furthermore, cell fate and tissue development in the embryo is regulated by a small number of key signaling molecules including; fibroblast growth factor (FGF), bone morphogenetic protein (BMP), nodal and sonic hedgehog (SHH). Whereas, bmp is important for dorsal-ventral axis formation **(Kudoh *et al*., 2004)**. BMP is involved in tail somite , as it expresses at gastrula stage ventrally **(Kwon *et al*.,2010)**.Additionally, bmp binds to Alk4/5 receptor and activates Smad1/5 and Smad4 leading the ventral specific gene activation at gastrula stage **(Mohedas *et al*., 2013).** Notably, the loss of bmp2b in zebrafish results in a swirl mutant phenotype that is characterized by the expansion of neural plate, notochord and somites with reduction of the epidermis and tail **(Kishimoto *et al*., 1997)**.In addition, snailhouse mutant, that is caused by bmp7 mutation, also results in strongly dorsalised embryo **(Dick *et al.,* 2000)**. It is worthmentioning that **Thorimbert *et al*. (2015)** identified DM arrested regeneration in the fin after blastemal formation in the zebrafish.

Thisse and Thisse (2005) cited that FGF and nodal contribute in mesoderm induction at early stage of development.In fact, FGF is important in mesoderm and neural induction **(Kudoh *et al*., 2004).** Hence, FGF function was detected through blocking it by the chemical inhibitor of SU5402. On the other hand, FGFs genes have 4 receptors, FGFR1, 2, 3 and 4, sending signals via Ras-MapK pathway **(Thisse *et al*., 1995).** Thus, it is considered a master regulater for mesoderm signaling. Moreover,the nodal pathway is important for mesoderm and endoderm formation and it can be blocked by SB inhibitor SB505124 **(Poulain *et al*., 2006)**. Nodal is a member of TGF-β family: nodal molecules mediate signals through binding with receptors Alk4, Alk5, and Alk7 that phosphorylate Smad2 and Smad3 **(Carcamo *et al*., 1994; Ryden *et al*., 1996).**

The sonic hedgehog (*shh*) signaling is emanated in notochord domain that is important for slow muscle induction **(Ochi & Westerfield, 2007).** Additionally, sonic hedgehog codes for SHH protein, and is under the family called hedgehog that also includes desert hedgehog(DHH) and Indian hedgehog(IHH). During embryonic development, Shh binds to patched and smoothened then it is released from patched. Smoothened subsequently initiates downstream signaling, leading the binding of Gli transcription factors to DNA and activates the gene expression **(Ochi & Westerfield, 2007; Lee *et al*., 2014).** Furthermore, *Shh* in zebrafish is expressed at 50% epiboly in the

organizer, and later in the notochord, in the floor plate and ventral brain **(Currie & Ingham, 1996; Ochi & Westerfield, 2007)** . While, hedgehog signaling plays a crucial role in muscle development. In addition, *Shh* plays a significant role in the patterning of different organs and tissues, including the limbs, skin, spinal cord, lung, hair, eye, somites, CNS, bones and testes **(Hammerschmidt *et al*.,1997; Murone *et al*.,1999; Perron *et al*., 2002; Prykhozhij & Neumann, 2008).** In zebrafish, it was found that blocking of *Shh*, using cyclopamine, results in inhibition of normal differentiation of muscle pioneers that organize the myotome of somite to give the muscle a normal shape **(Wolff *et al*., 2003).** *Shh* signaling that emanate from notochord is important to induce slow muscle **(Ochi & Westerfield, 2007; Lee *et al*., 2014).** These are all important signaling pathways and potentially important for mesoderm development. Hence, by using specific inhibitors of these signaling pathways, the key gene function and signaling pathway involved in the muscle development can be comprehended. Based on this hypothesis, an experimental plan was organized to treat embryos with chemical inhibitors that affect these pathways (DM, Cyclopamine , SU, and SB). Therefore, the aim of current study is to understand the key signaling pathways and molecular mechanisms that regulate mesoderm development .

MATERIALS AND METHODS

Zebrafish Danio rerio rearing

Danio rerio were reared in laboratory conditions similar to their natural habitat (28°C,7.3 pH), with water system supplied with oxygen pumps during 24 hours. Dried fry food was given to the larvae, and was replaced with alive artemia after 3 weeks. Cleaning and maintenance were carried out once a week. Fish laid eggs once or twice in a few weeks and deposited eggs in the day. To capture images with details, chorion was enzymatically removed using pronase (Sigma-Aldrich) (1mg/ml in Ringer). Embryos were anaesthetised at 24hpf with tricaine solution (0.004%) and then were photographed. This work was carried out during April 2019.

In situ probe preparation

One probe was made for in situ hybridization including Dr-*myl10*. Sp6 and T7 primers that were used for PCR. The PCR products, purified by phenol chloroform extraction, were ethanol precipitated. Antisense RNA probe was synthesized with T7 RNA polymerase (promega) and Dig-RNA labelling mix (Roche).

In situ hybridization staining

Chorion of *D.rerio* embryos were removed by pronase (Sigma-Aldrich) (1mg/ml in Ringer) and manually using tweezers. Then, they were fixed in 4% PFA/PBS and kept at 4°C for a minimum period of 5 days for fixation. Samples then were washed with 100% methanol and stored in -20°C at least for an hour, then washed in PBS tween (PBS,0.1% Tween 20) for 10 minutes. PBTW then was replaced with Hyp buffer solution, and the

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embryos were incubated for one hour in 65°C. Embryos after that were placed in preheated prop/Hyp buffer (90°C for 10 minutes and immediately in ice for 5 minutes) then kept in 65°C overnight. On the next day, the embryos were washed with a series of buffers : 50% formamide, 2X saline-sodium citrate (SSC),0.1% tween20 65°C, 30 minutes ; 2X SSC 0.1% tween20 65°C,30 minutes; 0.2XSSC0.1% tween20 65°C, 30 minutes twice and PBST at RT for 5 minutes. Embryos were blocked in a block solution +5% normal goat serum (NGS) for one hour, and replaced by an anti-dig antibody (1/100 dilution of 5000X) for 2 hours. Then, the embryos were washed 4 times each 30 minutes in a shaker, transferred into 24 well plates and treated with AP buffer for 10 minutes. Then, the AP buffer was replaced by BM-purple and kept in a dark box until stained. Finally, a 4% PFA/ PBS was used to stop staining and fix the embryos.

Treatment of *D.rario* with chemical inhibitors

Dorsomorphin (DM) (BMP inhibitor), SB 431542(nodal inhibitor), SU5402(Fgf inhibitor), Cyclopamine (sonic hedgehog inhibitor)) were applied on zebrafish *D.rario* at 1-2 cells stage (30 minutes post fertilization) and late gastrula (8 hpf) with DM,1-2 cells stage and sphere stage (4 hpf) with cyclopamine,1-2 cells stage and dome stage(4½ hpf) with SB431542, 1-2cells stage and 50%epiboly stage (5¼ hpf) with SU5402. The concentrations of DM were: 0 µM(control), 5 µM,10 µM and 20 µM; SB431542 were 0 µM(control), 25 µM,50 µM and 100 µM; SU5402 were 0 µM(control), 10 µM, 20 µM and 40 µM; and cyclopamine were 0 µM(control),50 µM and 100 µM. Three replications were used of each concentration (5 embryos in one well). Then, the embryos of *D.rerio* were cultured in 28°C for 24 hours, and chorions were removed by pronase (Sigma-Aldrich) (1mg/ml in Ringer). Afterwards, photographs were taken using Nikon SMZ1500. The embryos were fixed with 4%PFA/PBS (5 days) for in situ hybridization. Concentrations in the current study depeded on previous practice experiments, and it was found that the current concentration was more effective.

RESULTS

Treatment of the zebrafish with chemical inhibitors

To identify and characterise the key signaling pathways by which mesoderm development is regulated, several inhibitors were tested that block key signaling pathways in early embryonic development. The tested chemicals included: bone morphogenetic protein inhibitor (DM), Fgf inhibitor (SU5402), nodal inhibitor (SB431542), and sonic hedgehog inhibitor.

Nodal inhibitor (SB), with concentration 50µM in 1-2 cell stages, showed abnormal head and trunk (Fig.1B), whereas at 100µM a disappearance of the yolk and abnormality in the interior part of the body with short tail were observed (Fig.1C). At the dome stage of 50µM, a depression of the brain was monitored, while at 100µM an observation of a smaller head and a shorter tail were detected (Fig.1D). SU inhibitor expressed a short tail

morphology at concentration 10μM and 20μM that were exposed in 1-2cells (Fig.1E,F), additionally, all fish died at 40μM in the same stage.

Cyclopamine (shh inhibitor) caused supressed head parts and showed short body at concentrations 50μM and 100μM in 1-2cells stage and sphere stage (Fig.1G,H,I). At late gastrula stage of concentrations 5μM, 10μM and 20μM, DM presented short tail (Fig.1J), and all fish that were exposed to DM 5μM,10μM and 20μM at 1-2 cells stages died after 24hpf from exposure.
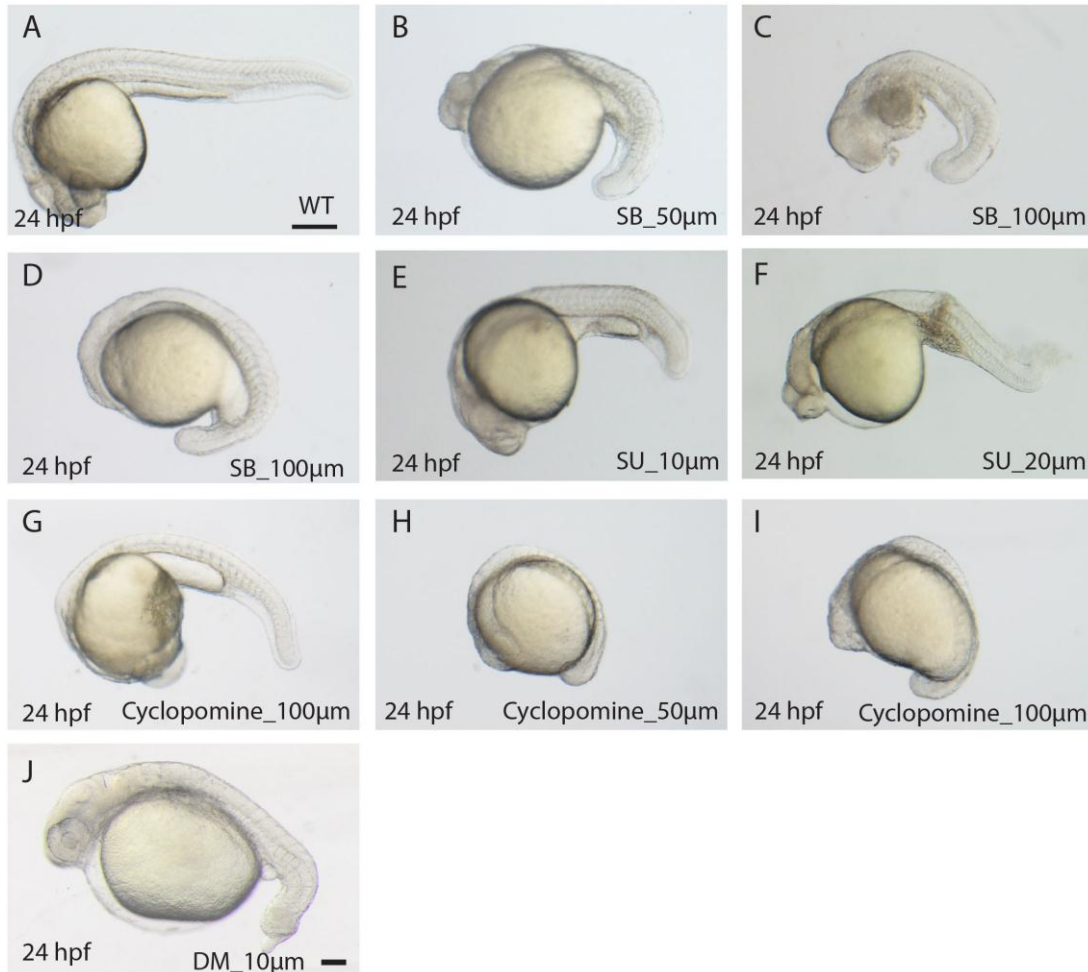

Fig.1 Live embryos of *D. rario* treated with chemical inhibitors .

A, wild type (24hpf). B, SB50μM (24hpf). C, SB100 μM(24hpf) (B and C , embryos treated at 1 cell stage) .D, SB100 μM (24hpf), embryos treated at dome stage. E, SU 10μM (24hpf).F, SU20 μM (24hpf)(E and F, embryos treated at 1 cell stage). G, Cyclopamine 100μM (24hpf) embryos treated at 1 cell stage. H, Cyclopamine 50μM (24hpf).I, Cyclopamine100 μM(24hpf). H and I, embryos treated at sphere stage. J, DM10 μM(24hpf), embryos treated at late gastrula. hpf (hour post fertalization). The scale bar = 100μM.

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In situ hybridization

The embryos of zebrafish treated with DM (5µM and 10µM) at late gastrula stage generated short tail embryo (Fig.2B, C), and DM showed deformity in the posterior part of the body at concentration 20µM at late gastrula stage (Fig.2D). Myosin light chain *(myl10)* stained the muscle for the embryos treated with DM at concentration 5µM and 10µM, while at 20µM concentration the *myl10* expression was lost and only a small part of deformed tail was stained. SB at concentration 50µM in 1-2 cell stage showed a small reduction in *myl10* (Fig.3B), but SB showed more reduction of the muscle at 100µM for the embryos treated at 1-2 cells stage (Fig.3C). At all concentrations of SU in both one cell stage and the 50% epiboly , *myl10* showed reduction in expression (Fig.4B-E) but it was more severe at 40µM when exposed from epiboly stage (Fig.4E). Cyclopam ininhibtor reduced *myl10* in embryos that were treated with 50µM one cell stage (Fig.5B), whereas at 100µM, *myl10* no expression was detected in the embryos that were exposed at one cell stage or sephere stage (Fig.5 Cand D).

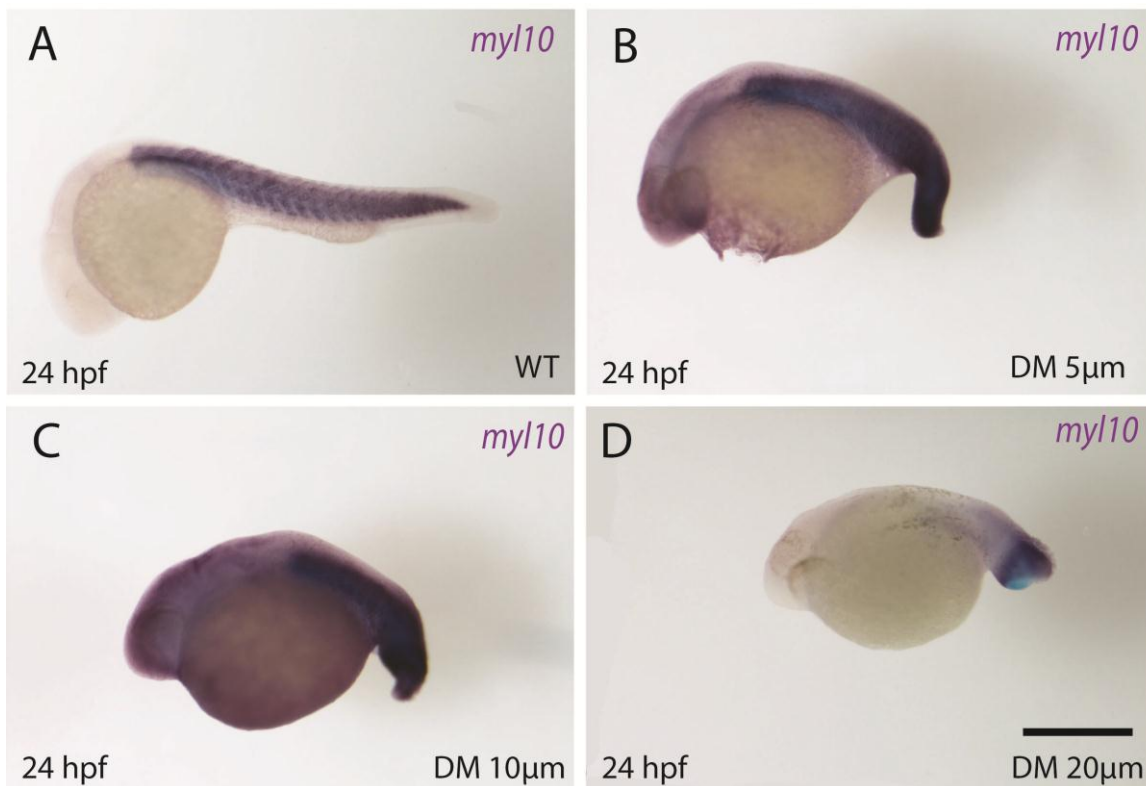**Fig.2 In situ hybridizaton with *my10* of *D.rerio* embryos treated with DM**.

 A, wild type. B, DM(5 µM). C, DM(10 µM). D, DM(20 µM).B-D embryos treated at late gastrula, they showed reduction of posterior part .D showed more severe in reduction of *myl10*. Scale bar=100µM.
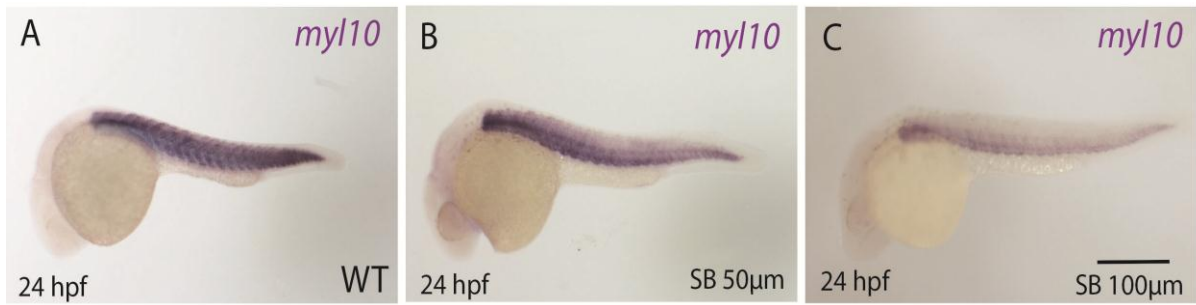
Fig. 3 In situ hybridizaton with *my10* of *D.rerio* embryos treated with SB.

A, wild type. B, SB(50 μM). C, SB(100 μM). B and C embryos treated at the one cell stage. SB reduced the gene marker *myl10* in trunk and tail muscles . Scale bar=100μM.
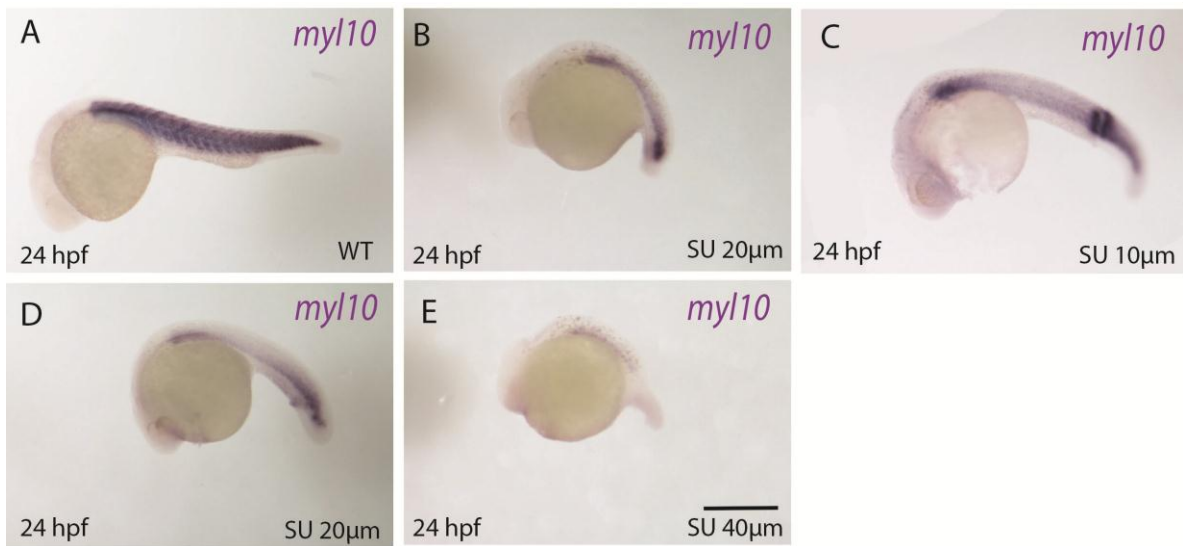

Fig.4, In situ hybridizaton with *my10* of *D.rerio* embryos treated with SU. A, wild type. B, SU(20μM) embryos treated at 1 cell stage. C,D and E SU(10μM, 20μM and 40μM respectively, embryos treated at 50% epiboly). B-E showed the reduction of *myl10* expression with more severer in E. Scale bar=100μM.
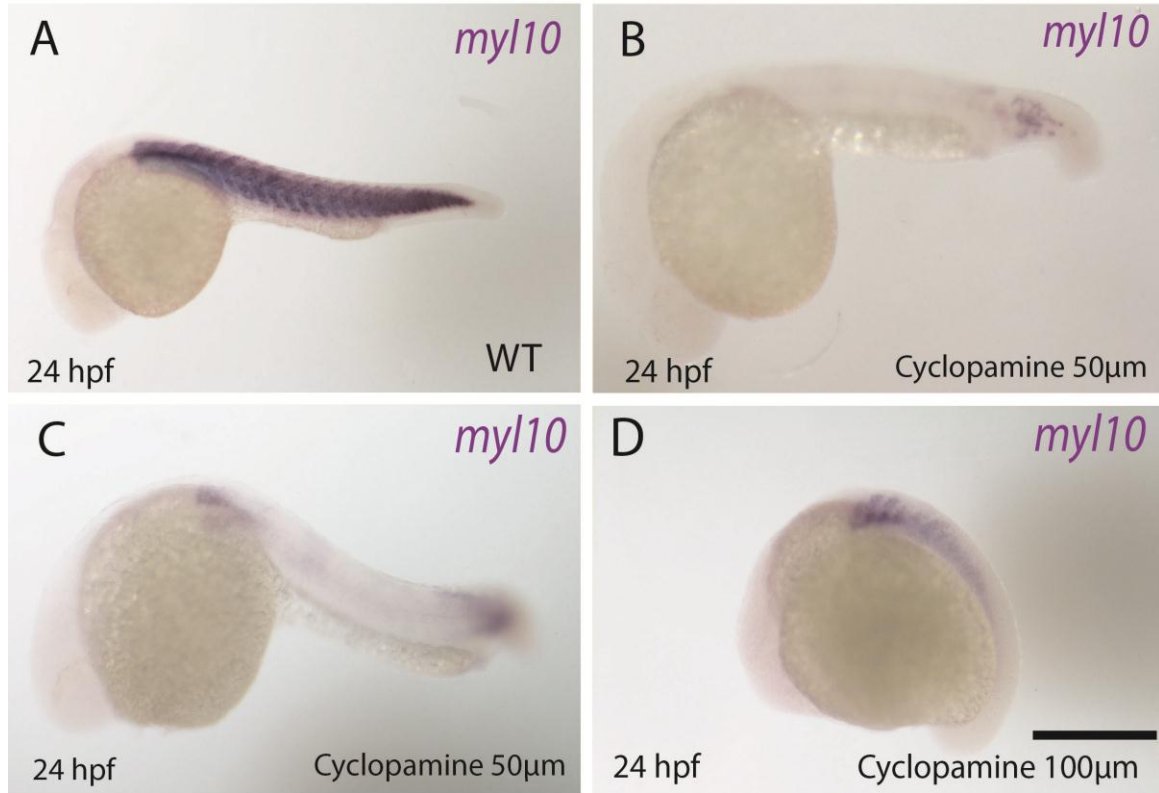
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Fig.5 In situ hybridizaton with *my10* of *D.rerio* embryos treated with Cyclopamine.
A, wild type. B, Cyclopamine (50μM) embryos treated at one cell stage. C, Cyclopamine (50μM). D, Cyclopamine (100μM). C and D, embryos treated at sphere stage. Cyclopamine inhibitor reduced *my10* in posterior part of embryo. Scale bar=100μM

Table (1) shows concentrations of inhibitors used in experiment for embryos at the stage of treatment and the percentage of abnormal and dead embryos. These results suggest that all tested signaling pathways are somehow involved in the development of mesoderm, showing the significant role of some transcription factors in the mesoderm induction but in different ways and different parts of the embryo through a different stage of development.

Table (1) Treatment of *D. rerio* with chemical inhibitors

Chemical	Concentration	Stage of treatment	Number (total)	Number (dead)	Number (abnormal)	% of abnormality
Dorsomorphin	5μm	1-2 cell	15	All dead	-	-
		Late gastrula	15	-	15	100
Dorsomorphin	10μm	1-2 cell	15	All dead	-	-
		Late gastrula	15	12	3	20
Dorsomorphin	20μm	1-2 cell	15	All dead	-	-
		Late gastrula	15	9	6	40
SU5402	10μm	1-2 cell	15	-	15	100
		50%epiboly	15	-	15	100
SU5402	20μm	1-2 cell	15	-	15	100
		50%epiboly	15	-	15	100
SU5402	40μm	1-2 cell	15	All dead	-	-
		50%epiboly	15	1	14	93.33
SB431542	25μm	1-2 cell	15	2	13	86.66
		Dome stage	15	-	15	100
SB431542	50μm	1-2 cell	15	3	12	80
		Dome stage	15	-	15	100
SB431542	100μm	1-2 cell	15	6	9	60
		Dome stage	15	-	15	100
cyclopamine	50μm	1-2 cell	15	4	11	73.33
		Sphere stage	15	2	13	86.66
cyclopamine	100μm	1-2 cell	15	4	11	73.33
		Sphere stage	15	3	12	80

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DISCUSSION

To use *D. rerio* as a new model species for studying the development of mesoderm, the current study was conducted to specify the role expression of some genes in early development of mesoderm induction. Thus, to attain this purpose a unique fish model was used to investagate the relationship among some genes and the early development in a relatively short time compared to other species such as *Cyprinus carpio* . It is clear that from the following embryonic development stages, the total period from fertilization to hatching that took 2-3 days **(Kimmel *et al.*, 1995)**, is considered short compared to other species like mangrove killifish *Kryptolebias marmoratus* recording 13 days as a hatching period **(Mourabit, *et al.*, 2011)**.

Treatment of *D .rario* with chemical inhibitors

To learn the roles of important signaling pathways in mesoderm development, zebrafish *D. rerio* embryos were treated with chemical inhibitors for candidate pathways. Considerably, DM is a specific inhibitor for Bmp (bone morphogenetic protein) pathway. Hence, Bmp is important for dorsal-ventral axis formation **(Kudoh *et al.*, 2004)**, especially that Bmp inhibitor (DM) suppressed the dorsal and ventral tissues. One possibility may be expressed is that Bmp inhibitor might suppress ventral and dorsal tissues formation and suppress the surrounding tissues that generate tail via affecting the gene activity of tail organizer. For instance, one of the zebrafish mutant that is called swirl is characterized by a reduction in the tail and epidermis due to the loss of bmp2b **(Kishimoto *et al.*, 1997)**. Moreover, the body axis of *K.marmoratus* are defected by BMP inhibitor **(Mourabit *et al.*, 2014)**.

Myosin protein expressed early in zebrafish, the expression of which is found present at the first day post fertilization **(Saud *et al.*,2021).** Myosin light chain (*myl10*), that is one of the important genes for slow muscles, is used as a marker to detect the role of mesoderm induction through inhibition of BMP. It was found that in situ hybridization supressed the posteriorir part of the body, and the deformation was more severe at concentration 20μM from late gastrula of exposure in a way that confirmed the important role of BMP during gastrulation. Since nodal and FGF are secretory protiens that induce mesoderm **(Thisse & Thisse, 2005)** thus, nodal inhibitor (SB431042) was used to suppress mesoderm formation by which mesoderm derived tissue such as muscle can be reduced. Though, in situ result showed mild reduction of *myl10* at 50μM in one cell stage, the reduction was more significant at concentration 100μM . The FgF inhibitor (SU5402) reduced spinal cord and muscle development because FGF has important role during different stages of embryonic development including one cell stage, as FgF ihhibitor was applied in one cell stage and 50% epiboly. In one cell stage of exposure, the tail became short and *myl10* marker exhibited a reduction of muscles, while the severity

was more clear in muscle reduction at concentration of 40μM. These are all important signaling pathways that are essential for mesoderm development. By using specific inhibitors of those signaling pathways, the effect of these pathways on mesoderm development might be easy to investigate.

Therefore, cyclopamine (Sonic hedgehogh inhibitor) can reduce the signaling emanating from the midline around the notochord. In addition, *Shh* is important for specifying different somite muscle types. In the current study, cyclopamine changed the position of the muscles and reduced *myl10,* and the severity of defection was observed at the concentration of 100μM confirming their role in muscle induction.

The present study suggest that many signaling pathways are important in mesoderm development. However, it is possible that these pathways have crucial roles in early lineage specification (e.g. mesoderm induction). To further characterise the signaling pathways, it is needed to examine other lineage markers (e.g. fast muscle).

CONCLUSION

Many inhibitors (DM, SU, SB and Cyclopamine) were tested and concluded that these chemicals inhibit mesoderm development. Yet, further analysis using more markers and stages with specific chemical treatments are required as well as using knock down and knock out gene technique that can show deep details for the role and time expression of genes in different stages during mesoderm induction.

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