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The MEK-ERK signaling pathway promotes maintenance of cardiac chamber identity

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The zebrafish heart is comprised of two distinct chambers

The vertebrate heart is comprised of two types of chambers, ventricles and atria, which exhibit unique structural and contractile properties due to chamber-specific differences in gene expression. It is therefore important for ventricular and atrial cardiomyocytes to establish and maintain their particular cellular characteristics.

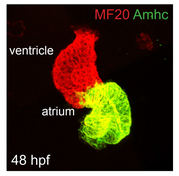


Figure 1. The zebrafish heart is comprised of two distinct chambers, a ventricle and an atrium. Immunofluorescence with MF20 (recognizes myosin heavy chain, marks the whole myocardium) and S46 (recognizes Amhc). At 48 hours post fertilization (hpf), the ventricle and the atrium are morphologically distinct.

FGF signaling is required for maintenance of ventricular chamber identity

Our recent work demonstrated that FGF signaling enforces cardiac chamber identity in the developing ventricle. When FGF signaling is inhibited after the initiation of myocardial differentiation, ventricular cardiomyocytes begin to ectopically express *atrial myosin heavy chain* (*amhc)* (Fig. 2), representing a transformation of ventricular cells to cells with atrial characteristics. However, the molecular mediators that act between the reception of FGF signals and the regulation of chamber-specific gene expression patterns remain unclear.

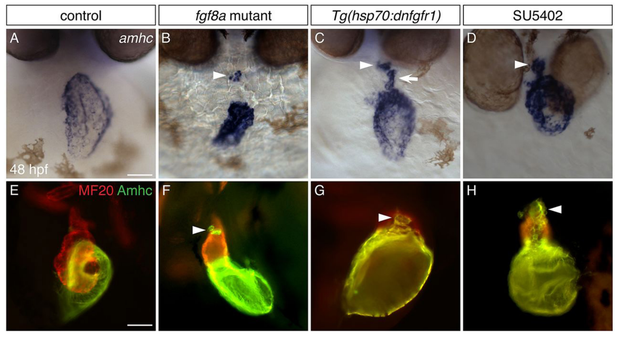


Figure 2. FGF signaling is required to maintain ventricular chamber identity. (A-D) *amhc* in situ hybridization in frontal views at 48 hpf. (A) In control embryos, *amhc* is restricted to the atrium. In *fgf8a* mutants (B), *amhc* is ectopically expressed in a small group of ventricular cells near the arterial pole (arrowhead). More ectopic *amhc* is observed near the arterial pole (arrowhead) and in the inner curvature (arrow) in *Tg(hsp70:dnfgfr1)* embryos heat shocked at 18 hpf (C) and embryos treated with 5 μM SU5402 (FGFR inhibitor) starting from 18 hpf (D). (E-H) Immunofluorescence with MF20 and S46 at 48 hpf. (E) In control embryos, Amhc is restricted to the atrium. In *fgf8a* mutants (F), *Tg(hsp70:dnfgfr1)* embryos heat shocked at 18 hpf (G) and embryos treated with 5 μM SU5402 from 18-30 hpf (H), ectopic Amhc is observed in the ventricle (arrowheads). Modified from Pradhan et al., 2017.

MEK-ERK signaling represses *amhc* expression in ventricular cardiomyocytes

To determine which pathways downstream of FGF regulate maintenance, we examined the effects of pharmacological inhibitors. Inhibition of either the JNK pathway or the PI3K pathway failed to induce ectopic *amhc* in the ventricle (data not shown). In contrast, inhibition of MEK1/2-ERK1/2 signaling using PD0325901 induced ectopic *amhc* expression in the ventricle (Fig. 3), suggesting that MEK-ERK signaling reinforces ventricular identity. Similar to FGF inhibition (Fig. 3C,F), the effects of MEK inhibition were very potent between 18 to 26 hpf (Fig. 3B,E), and its potency decreased significantly with treatment starting at 26 hpf (Fig. 3H,I), suggesting overlap between the time intervals when MEK-ERK signaling and FGF signaling are required.

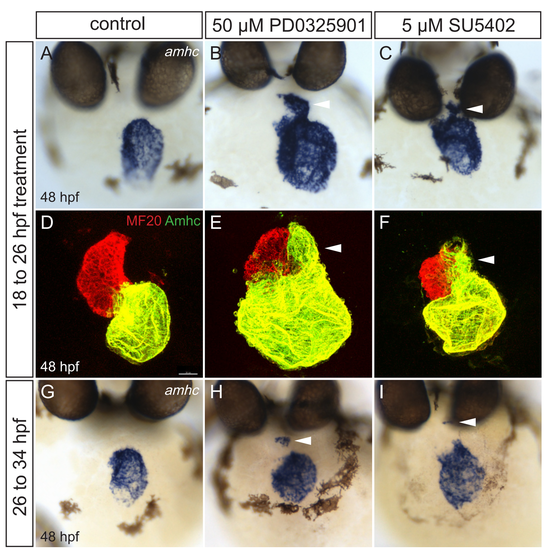


Figure 3. MEK1/2 inhibition induces ectopic *amhc* expression in the ventricle in a similar time interval as FGF inhibition. Embryos were treated from 18 to 26 hpf (A-F) or from 26 to 34 hpf (G-I). (A-C, G-I) *amhc* in situ hybridization in frontal views at 48 hpf. (D-F) Immunofluorescence with MF20 and S46 at 48 hpf. There are comparable distributions of ectopic *amhc* in the ventricle (arrowheads) in embryos treated with PD0325901 (B,E) or SU5402 (C,F). Initiation of treatment at 26 hpf induces less ectopic *amhc* in the ventricle (arrowheads) (H,I).

MEK-ERK signaling promotes *vmhc* expression in ventricular cardiomyocytes

As another similarity with the effects of FGF inhibition (Fig. 4C), we found that MEK inhibition results in a reduction of the expression of *ventricular myosin heavy chain* (*vmhc*) in the ventricle (Fig. 4B), suggesting that MEK-ERK signaling, like FGF signaling, promotes ventricular gene expression.

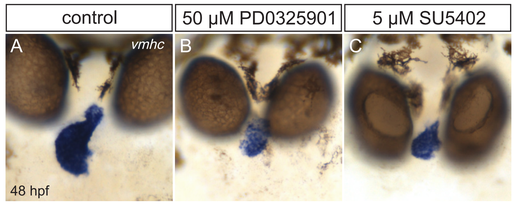


Figure 4. MEK1/2 inhibition results in a reduction of *vmhc* expression in the ventricle. Embryos were treated from 18 to 26 hpf. (A-C) *vmhc* in situ hybridization in frontal views at 48 hpf. (A) In control embryos, *vmhc* expression is found in the ventricle and the outflow tract. PD0325901-treated embryos (B) and SU5402-treated embryos (C) display a smaller area of *vmhc* expression, as well as reduced intensity of expression.

Effective reduction of FGF signal transduction with MEK1/2 inhibition

Under our treatment conditions, SU5402 and PD0325901 reduced the expression of a FGF activity reporter, *Tg(dusp6:d2egfp)*, to a similar level (Fig. 5), indicating effective reduction of FGF signaling by PD0325901.

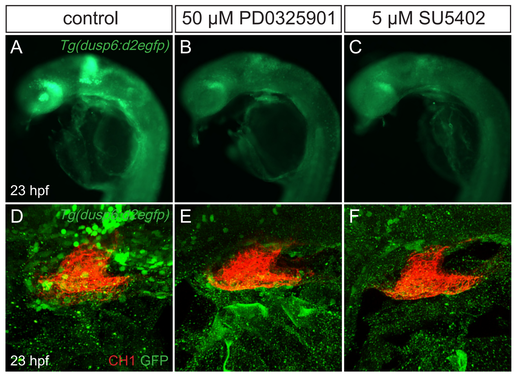


Figure 5. Treatment with SU5402 or PD0325901 reduced FGF activity to a similar level. Embryos were treated from 18 to 23 hpf. (A-C) Immunofluorescence with anti-GFP at 23 hpf. Compared to control embryos (A), expression of *Tg(dusp6:d2egfp)* is reduced in both PD0325901- (B) and SU5402- (C) treated embryos. (D-F) Immunofluorescence with anti-GFP and CH1 (anti-tropomyosin, marks myocardium) at 23 hpf. *Tg(dusp6:d2egfp)* is expressed in some cells of the heart tube in control embryos (D). This expression is reduced in both PD0325901- (E) and SU5402- (F) treated embryos.

nkx factors act downstream of FGF-ERK signaling during ventricular maintenance

The transcription factor genes *nkx2.5* and *nkx2.7* act to maintain ventricle-specific gene expression patterns. Previously, we found that FGF acts through *nkx* factors to promote ventricular maintenance, as *nkx2.5* overexpression results in a partial rescue of phenotypes caused by reduction of FGF signaling (Fig. 6). Similar to FGF signaling, as reported before (Fig. 7A-D), MEK signaling promotes *nkx* gene expression (Fig. 7E-H), suggesting that ERK signaling might act as the mediator between FGF signaling and *nkx* factors.

Condition	Number of embryos with ectopic *amhc*	Total number of embryos	% embryos with ectopic *amhc*
nontransgenic	65	77	84%
Tg(hsp70:nkx2.5)	76	119	64% *

Figure 6. *nkx2.5* overexpression results in a partial rescue of phenotypes caused by FGF inhibition. Embryos were treated with SU5402 at 18 hpf and heat shocked at 24 hpf. Compared to nontransgenic siblings (84%), fewer embryos exhibited ectopic *amhc* when *nkx2.5* overexpression is induced by heat shock (64%). Asterisk indicates statistical significance (p=0.0019, Fisher's exact test). Modified from Pradhan et al., 2017.

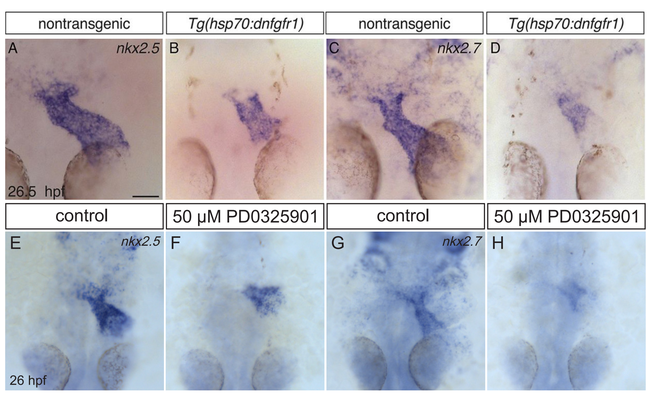


Figure 7. FGF or MEK1/2 inhibition results in reduction of *nkx* expression in the heart tube. (A-D) Embryos were heat shocked at 18 hpf. (E-H) Embryos were treated from 18 to 26 hpf. *nkx2.5* (A-B, E-F) and *nkx2.7* (C-D, G-H) in situ hybridization in dorsal views. In control embryos, *nkx2.5* (A, E) and *nkx2.7* (C, G) is robustly expressed in the heart tube, while in FGF-inhibited or PD0325901-treated embryos, *nkx2.5* (B, F) and *nkx2.7* (D, H) expression is greatly reduced. (A-D) are modified from Pradhan et al., 2017.

Different regions of the ventricle exhibit differential degrees of plasticity

Not all of the cells in the ventricle are equally sensitive to MEK or FGF inhibition. Lower levels of MEK inhibition typically induce ectopic *amhc* in the outflow tract (OFT) and the inner curvature (IC) (Fig. 8B), while higher levels of inhibition induce broader expression throughout the ventricle (Fig. 8C-F). This suggests that ventricular cells are not equally plastic.

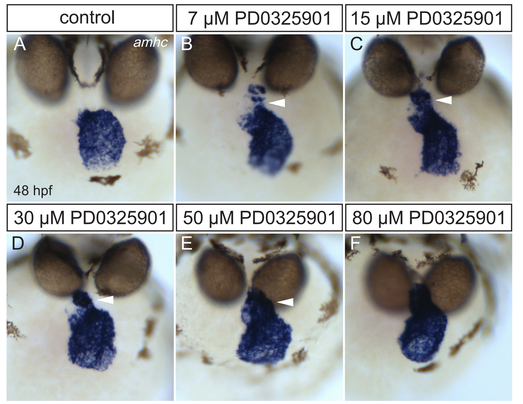


Figure 8. Different regions of the ventricle are not equally sensitive to MEK inhibition. Embryos were treated from 18 to 43 hpf. (A-F) *amhc* in situ hybridization in frontal views at 48 hpf. (A) In control embryos, *amhc* expression is restricted to the atrium. (B-E) Embryos treated with PD0325901 exhibit ectopic *amhc* near the arterial pole, in the inner curvature (arrowheads) as well as in outer portions of the ventricle. (F) All of the ventricular cardiomyocytes in 80 μM PD0325901-treated embryos express ectopic *amhc*.

Regional differences in exposure to other signaling pathways may confer differential degrees of plasticity

As FGF activity in the linear heart tube, as demonstrated by expression of *Tg(dusp6:d2egfp),* shows no obvious regionalized pattern, regional differences in gene expression or exposure to other signaling pathways may determine the level of FGF-ERK signaling needed for ventricular maintenance. BMP pathway activity appears enriched to the pre-IC region as early as 30 hpf (Fig. 9). Additionally, we have found that activation of BMP signaling can lead to mild ectopic *amhc* expression (Fig. 10B and data not shown), suggesting that limiting the level of BMP signaling might be important for maintenance of ventricular identity.

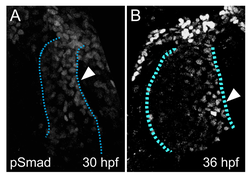


Figure 9. Enriched BMP activity in pre-IC region. Immunofluorescence of pSmad at 30 (A) and 36 hpf (B). Higher levels of pSmad are detected in the IC (arrowheads) than in the OC of the ventricle. Images by Dena M. Leerberg.

To test this hypothesis, we used *Tg(hsp70l:ca-alk6)* to activate BMP signaling before 18 hpf. In the context of MEK inhibition, embryos with activation of BMP signaling (Fig. 10D) exhibited more ectopic *amhc* expression than those without (Fig. 10C), suggesting that BMP activation enhances the effects of MEK inhibition.

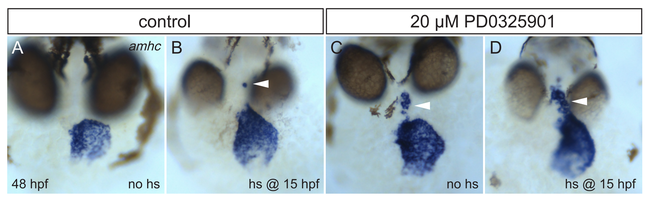
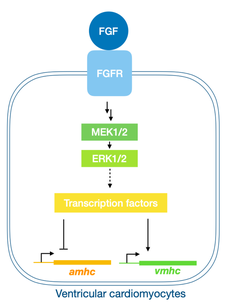


Figure 10. BMP activation enhances the effects of MEK1/2 inhibition in inducing ectopic *amhc* in the ventricle. *Tg(hsp70l:ca-alk6)/+ embryos* were treated from 18 to 26 hpf. (A-D) *amhc* in situ hybridization in frontal views at 48 hpf. In DMSO-treated embryos without heat shock (A), *amhc* is restricted to the atrium, while in those heatshocked at 15 hpf (B), *amhc* expression can be observed in one or two cells in the ventricle (arrowhead). In PD0325901-treated embryos without heat shock (C), ectopic *amhc* is most frequently observed in the OFT and some parts of the IC (arrowhead), while in PD0325901-treated embryos that were heat shocked at 15 hpf (D), ectopic *amhc* is most frequently observed in the OFT and the entire IC (arrowhead).

Model and Future Directions



Together, our studies support a model in which MEK-ERK signaling, activated by upstream FGF signaling, is required after the onset of cardiomyocyte differentiation to promote ventricular characteristics and suppress atrial characteristics in ventricular cardiomyocytes.
It is interesting to note that not all ventricular cells require the same levels of MEK-ERK signaling to maintain their identity. Different endogenous levels of BMP signaling may contribute to these regional distinctions within the ventricle.

Figure 11. Model illustrating the molecular mechanisms underlying the maintenance of ventricular chamber identity.

In the future, we are interested in addressing the following open questions:

- Is MEK-ERK signaling the primary pathway acting downstream of FGF signaling in ventricular maintenance?
- When and where is MEK-ERK signaling required for ventricular maintenance?
- What are the downstream effectors of ERK signaling that complement *nkx* factors during maintenance?
- Is BMP signaling the factor that confers differential degrees of plasticity within distinct regions of the ventricle?