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Extracellular component hyaluronic acid and its receptor Hmmr are required for epicardial EMT during heart regeneration

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Aims After injury, the adult zebrafish can regenerate the heart. This requires the activation of the endocardium and epicardium as well as the proliferation of pre-existing cardiomyocytes to replace the lost tissue. However, the molecular mechanisms involved in this process are not completely resolved. In this work, we aim to identify the proteins involved in zebrafish heart regeneration and to explore their function.

Methods and results Using a proteomic approach, we identified Hyaluronan-mediated motility receptor (Hmmr), a hyaluronic acid (HA) receptor, to be expressed following ventricular resection in zebrafish. Moreover, enzymes that produce HA, hyaluronic acid synthases (*has*), were also expressed following injury, suggesting that this pathway may serve important functions in the regenerating heart. Indeed, suppression of HA production, as well as depletion of Hmmr, blocked cardiac regeneration. Mechanistically, HA and Hmmr are required for epicardial cell epithelial–mesenchymal transition (EMT) and their subsequent migration into the regenerating ventricle. Furthermore, chemical inhibition of Focal Adhesion Kinase (FAK) or inhibition of Src kinases, downstream effectors of Hmmr, also prevented epicardial cell migration, implicating a HA/Hmmr/FAK/Src pathway in this process. In a rat model of myocardial infarction, both HA and HMMR were up-regulated and localized in the infarct area within the first few days following damage, suggesting that this pathway may also play an important role in cardiac repair in mammals.

Conclusion HA and Hmmr are required for activated epicardial cell EMT and migration involving the FAK/Src pathway for proper heart regeneration.

Keywords Zebrafish heart regeneration • Hyaluronic acid • *Hmmr* • Epicardial cell migration • pFAK

1. Introduction

Ischemic heart disease is one of the most common causes of mortality in developed countries. After myocardial infarction (MI), billions of cardiomyocytes undergo apoptosis, pyroptosis, and necrosis, and a non-contractile collagen scar that limits the cardiac function is formed.[1] The ability of the mammalian heart to replace lost cardiomyocytes is limited.[2] Neonatal mice are able to regenerate after amputation of the ventricular apex,[3] and after MI,[4] but regeneration is restricted to post-natal day 7 (P7), after which the majority of cardiomyocytes become post-mitotic. More recently, cardiac ischaemic injury during the phase of cardiac growth in adolescent mice (P15) also exhibited regenerative capacity, suggesting that under certain conditions regeneration can occur in mammals.[5] In contrast, adult zebrafish (*Danio rerio*) can efficiently regenerate the heart after amputation of the ventricle apex throughout its lifespan.[6,7] A key factor of the regenerative process in zebrafish is the ability of pre-existing cardiomyocytes to undergo proliferation following organ damage.[8–11] Another feature is the activation and proliferation of epicardial cells to undergo epithelial–mesenchymal transition (EMT), followed by their migration into the injury site to promote angiogenesis.[9,12–14] In addition, within 3 h of ventricular injury, the endocardium undergoes morphological changes and induces the expression of retinoic acid (RA)-synthesizing enzyme *aldh1a2*, indicating that the endocardium is also a dynamic player in

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zebrafish heart regeneration.[13] The molecular pathways that direct these processes are beginning to be elucidated with evidence that fibroblast growth factors (FGF),[11,14] transforming growth factor-β (TGF-β),[15] platelet derived growth factor β (PDGFβ),[16] insulin-like growth factor 2 (IGF2),[17] RA,[13] Jak1/Stat3,[18] neuregulin 1 (Nrg1),[19] Hedgehog,[20] and Notch signaling[21] playing important roles to promote cardiomyocyte proliferation, endocardium activation, and epicardial EMT.

In this pilot study, we analysed the proteomic changes following cardiac resection of the ventricular apex in adult zebrafish. We identified increased expression of an hyaluronic acid (HA) receptor (Hmmr) and hypothesized that the HA pathway could play a crucial role in cardiac regeneration. HA is a large, linear, non-sulfated GAG component of extracellular matrix (ECM). Following injury, HA is produced in the inner side of the plasma membrane by HA Synthases (HAS) and is extruded onto the cell surface where it accumulates in the wound to promote cellular proliferation and migration to support tissue remodelling and healing.[22,23] Chemical suppression of HA synthesis as well as knockdown of Hmmr blocked cardiac regeneration. Mechanistically, we observed decreased migration of activated epicardial cells into the regenerating heart and reduced coronary vasculature, suggesting that HA is important for epicardial EMT. Our studies document the importance of HA and its receptor in epicardial cell migration into the clot tissue for remodelling of the nascent coronary vasculature.

2. Methods

2.1 Zebrafish maintenance, ventricular amputation, and retro-orbital injections

The zebrafish experiments were performed according to protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh that conforms to the NIH guidelines. Adult (6–18 months) wild type AB* and transgenic *Tg(myl7:EGFP)*^{f1},[24] *Tg(fli1a:EGFP)*^{y1},[25] *Tg(wt1b:EGFP)*^{li1} (a kind gift from Christoph Englert)[26] zebrafish were maintained at 28°C. Zebrafish were anaesthetized by immersing in 0.168 g/L ethyl 3-aminobenzoate methanesulfonate salt (MS-222; Sigma) for 3–5 min. Zebrafish were placed onto a wet sponge with the abdomen facing up. Approximately 20% of the ventricle apex was resected as described by Poss *et al.*,[6] and zebrafish returned to the system for recovery before retro-orbital injections of drugs or with *Vivo Morpholinos (VMO)* at one day post-surgery as described by Pugach *et al.*[27] Retro-orbital injections were performed daily for 2, 4, 6, or 10 days at which point hearts were extracted. To suppress HA production, zebrafish were injected with 3 μL of 500 μM 7-hydroxy-4-methylcoumarin (HMC) (Acros Organics) (also known as 4-methylumbelliferone, 4-Mu), or with PBS as vehicle control. To inhibit Focal Adhesion Kinase (FAK), 3 μL of 100 μM of PF-573228 (Sigma) dissolved in DMSO (Sigma) was injected. To suppress Src Kinase, 3 μL of 200 μM Src Inhibitor 1 (SKI-1) (Sigma) or vehicle DMSO was injected. To knockdown Hmmr, 1 μL of 3 mg/kg Hmmr E414 spliced *VMO* (Gene Tools, LLC) (TGTGCAAACAGATG TACCTCTTTCT) was injected. For controls, 5 bp mismatch *VMO* (Mut-MO) (TGAGGAAACACATCTACGTCTTTCT) was retro-orbitally injected.

2.2 Difference gel electrophoresis and MS/MS analysis

A detailed description of difference gel electrophoresis (DiGE) and MS/MS analysis is provided in the Supplementary material online.

2.3 *In situ* hybridization, immunostaining, clot area measurement, and cell counting

For histological examination, zebrafish were euthanized using 0.168 g/L ethyl 3-aminobenzoate methanesulfonate salt for 15 min, and hearts were collected in cold PBS and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Hearts were cryopreserved with sucrose before immersion in embedding media (Instrumedics). Fourteen micrometre cryosections were collected, and consecutive sections were used for *in situ* hybridization (ISH), immunostaining, and Acid Fuchsin Orange G (AFOG). ISH was performed in cryosections, using digoxygenin-labelled cRNA probes as described.[14] BM purple (Roche) was used as AP substrate. AFOG staining was performed as described by Poss *et al.*[6] Images were captured with Leica MZ 16 microscope and Q Imaging Retiga 1300 camera. A detailed description of clot area measurement and cell counting is provided in Supplementary material online.

2.4 RNA extraction, cDNA synthesis, PCR, and quantitative PCR

Total RNA was isolated from uninjured hearts and hearts at 1, 3, and 7 days post-amputation (dpa) using TRIzol reagent (Invitrogen), and RNeasy Micro kit (Qiagen), according to manufacturer's instructions. Eight hearts were pooled together for each condition. One microgram of total RNA was reverse transcribed to cDNA with SuperScript (Invitrogen) using random hexamers. PCR was performed to test the efficacy of *hmmr* Spliced V-MO knockdown. *Eukaryotic translation elongation factor 1 alpha 1, like 1* (*eef1a1l1*) was used as reference gene. The primers sequences for PCR are the following: *eef1a1l1*-F ATCTACAAATGCGGTGGAAT; *eef1a1l1*-R ATACCAGCCTCAAACTCACC; *hmmr*-Ex3-F GGACCATGTCTGTT GATGGTTTGGCTG; *hmmr*-Ex-7-R GACCTTTACCTTTCCTTCTG AGC. RT–PCR products were electrophoresed on agarose gels and stained with ethidium bromide. The primer sequences used for quantitative PCR (Q-PCR) were designed using Beacon designer and are listed in Supplementary material online, *Table S1*. Two step real-time PCR was performed using SYBR Green (Bio-rad) in a PCR system iQ5 thermal cycler (Bio-rad). Primers set efficiency was calculated and adjusted using LinRegPCR. β-Actin and RNA polymerase were used to normalize gene expression in the Q-PCR experiments. Experiments were done in triplicate.

2.5 Western blot

Proteins were extracted from a pool of five ventricles apex of each condition, using 200 μL of Laemmli Buffer (Bio-rad), containing β-mercaptoethanol (Fisher). Samples were heated at 95°C for 5 min, and 0.1 M dithiothreitol (Calbiochem) was added before loading. After SDS–PAGE, proteins were transferred in nitrocellulose membrane (Li-cor). Membrane was blocked in Odyssey Blocking Buffer (Li-cor) for 1 h. Antibodies were diluted in Odyssey Blocking Buffer, containing 0.2% Tween 20 (National diagnostics). Blots were scanned using Li-cor Odyssey CLx Infrared imaging system, and band intensity was quantified and normalized using Image Studio software. Primary antibody used were anti-Twist1 (Sigma) (1:1000), anti-ERK-2 (Sigma) (1:500), anti-actin (Sigma) (1:5000), and anti-FAK [pY397] (Invitrogen) (1:1000). Secondary antibody used for western blot was IRDye 800 donkey anti-rabbit IgG (H + L) (Li-cor) (1:15000), and IRDye 680 goat anti mouse (H + L) (Li-cor).

2.6 *Ex vivo* cell migration assay

Epicardial cell migration was measured in *ex vivo* assay as described by Kim *et al.*,[28] using *Tg(wt1b:EGFP)*^{li1} fish. Briefly, 3 dpa hearts were extracted from zebrafish retro-orbital injected for 2 days with HMC, *hmmr VMO*, PF-573228, SKI-1, or vehicles PBS and DMSO. Hearts were cultured in 24-well plate pre-coated with fibrin and incubated at 28°C for 3 days. Cell migration was measured using ImageJ from the edge of the heart to the edge of the cell monolayer at 1, 2, and 3 days post-extraction.

2.7 Rat maintenance and myocardial infarct induction

A detailed description of rat maintenance and MI induction is described in Supplementary material online.

2.8 Statistical analysis

Statistical significance was analysed by the Student's *t*-test, one-way ANOVA, and two-way ANOVA and shown as mean ± S.D. For one-way ANOVA, Tukey *post hoc* tests were performed. *P*-values were considered significant when <0.05.

3. Results

3.1 Atp5a1, desmuslin, and Hmmr are up-regulated after heart injury

To identify the proteins that are differentially expressed during zebrafish heart regeneration, we performed a pilot DiGE experiment on 3 dpa and control uninjured hearts (see Supplementary material online, *Figure S1A* and *B*). We picked the most prominent protein spots that were within the detection range of MALDI-TOF/TOF-MS for protein identification. We identified ATP Synthase 5a1 (Atp5a1), desmuslin, and hyaluronan-mediated motility receptor (Hmmr), to be increased in heart samples at 3 dpa (see Supplementary material online, *Figure S1A–C*). Previous work revealed the importance of HA in cardiac development and tail regeneration.[29,30] However, the role for HA receptor in regeneration is not known. Therefore, we sought to understand the function of Hmmr in zebrafish cardiac regeneration. We verified that *hmmr* gene transcription was also increased following injury by *in situ* hybridization (ISH) and Q-PCR. *hmmr* expression was absent in uninjured hearts but was markedly increased throughout the whole heart after injury (*Figure 1A*) and confirmed by Q-PCR (*Figure 1B*). A rapid increase in *hmmr* expression was observed at 3 dpa, but expression declined at 7 dpa (*Figure 1B*). Similarly, expression of *cluster of differentiation-44 (cd44)*, a co-receptor for HA, was induced at 3 and 7 dpa. Moreover, the enzymes that synthesize HA and HA synthases (*has1* and *has2)* were also up-regulated at 3 dpa (*Figure 1B*). In contrast, expression of *hyaluronidases2 (hyal2)*, an enzyme responsible for HA degradation, did not change, suggesting that upon injury HA accumulates in the heart.

To confirm the presence of HA in the heart, we used biotinylated HA binding protein (bHABP) to detect HA accumulation following cardiac injury.[31] In uninjured hearts, HA was not detectable, but at 3 and 7 dpa substantial HA was localized in cells adjacent to the wound and also within the clot (*Figure 1C*). By 14 and 30 dpa, the HA was still present, but it was restricted to the clot tissue (*Figure 1C*). Furthermore, immunostaining for Has proteins revealed that expression was restricted in the clot area and co-localized with HA, confirming that upon injury, HA accumulates within the damaged tissue (see Supplementary material online, *Figure S2A*).[32] Thus, Has proteins and HA receptors are rapidly induced following cardiac damage, suggesting that these factors are important in zebrafish heart regeneration.

3.2 HA is required for proper heart regeneration

To determine the importance of HA during cardiac regeneration, we suppressed HA synthesis after ventricular resection using HMC, an inhibitor of Has enzymes by depleting its cellular substrate, UDP-glucoronic acid.[33,34] HMC was delivered by retro-orbital injections following cardiac injury (*Figure 2A*). At 3 dpa, HA in the clot area was greatly diminished in HMC-injected fish (*Figure 2B–E* and see Supplementary material online, *Figure S2B* and *C*). By 30 dpa, when regeneration is complete[6] (*Figure 2F* and *H*), HMC-injected zebrafish still contained significant scar tissue (*Figure 2G* and *H*). In concordance with these observations, HMC-injected zebrafish contained activated myofibroblasts as indicated by the presence of α-smooth muscle actin (α-SMA) within the clot tissue at 15 dpa (*Figure 2I* and *J*). Activated myofibroblasts were even detected at 60 dpa, suggesting that blocking HA production can completely block the regenerative process resulting in permanent scar tissue deposited in the heart (see Supplementary material online, *Figure S3*).

To understand mechanistically how HA may function in cardiac regeneration, we measured cardiomyocyte proliferation in HMC-injected adults. At 7 dpa, the number of cardiomyocytes (Mef2c) in S phase (PCNA) was not statistically different from control PBS-injected zebrafish (see Supplementary material online, *Figure S4*). However at 10 dpa, significant reduction in cardiomyocytes inside the clot was observed compared with controls, implicating a deficiency in cardiomyocyte repopulating the clot tissue (*Figure 3A*, *B*, and *G*) that persisted at 60 dpa (see Supplementary material online, *Figure S5*).[10,35] The lack of cardiomyocytes in the clot tissue could reflect a migratory defect and their survival in HMC-injected hearts after the proliferative phase. Another important aspect of cardiac regeneration is the establishment of the coronary vasculature to support the regenerating cardiac tissue. Therefore, we next determined whether the formation of the coronary vasculature was also disrupted. HMC injections into a transgenic line that labels blood vessels with green fluorescent protein (*Tg(fli1a:EGFP)*) showed a significant reduction in coronary vasculature in the clot area (*Figure 3C–F* and *H*, and see Supplementary material online, *Figure S6*). Moreover, the endothelial cells that were detected in HMC-injected zebrafish were sparse and failed to organize into vessel-like structures. The lack of coronary vasculature was confirmed by immunostaining for the presence of Fli1b, another endothelial-expressed Ets-transcription factor important for vessel formation.[36] In HMC-injected zebrafish, there was a pronounced decrease in $Fli1b^{+}$ cells within the clot (*Figure 3I*, *J*, and *K*). Taken together, these results show that HA is produced following cardiac injury and plays a critical role in the formation of the coronary vasculature and in the repopulation of cardiomyocytes in the clot tissue.

3.3 *Hmmr* is necessary for heart regeneration

Given the importance of HA in heart regeneration, we next determined whether the Hmmr is also required in this process. *hmmr* was knocked down using *VMO*. *VMOs* have been shown to be effective in targeting the depletion of protein expression in several tissues in mice and in adult zebrafish.[37] The *hmmrVMO* was designed to suppress intron splicing between exons 4 and 5 (see Supplementary material online, *Figure S7A*). RT–PCR experiments confirmed that *hmmr* transcripts in pooled injured hearts were reduced in zebrafish injected with *hmmrVMO,* but not in control *hmmr-MutMO* (5-bp mismatch *VMO*) (see Supplementary material online, *Figure S7B*). Following ventricular resection, *hmmrVMO*, *hmmr-MutMO*, or PBS were retro-orbitally injected from 1 dpa until 10 dpa, and fish were allowed to recover until time of analyses. At 30 dpa, scar tissue area was significantly larger in *hmmrVMO*-injected zebrafish compared with PBS and to the *hmmr-MutMO* controls (*Figure 4A–D*). As noted with suppressing HA production, knockdown of *hmmr* also decreased angiogenesis at 10 dpa (*Figure 4E–K* and see Supplementary material online, *Figure S8*), without affecting cardiomyocyte proliferation (see Supplementary material online, *Figure S9*). Thus, depletion of *hmmr* resulted in decreased regenerative capacity of the zebrafish heart.

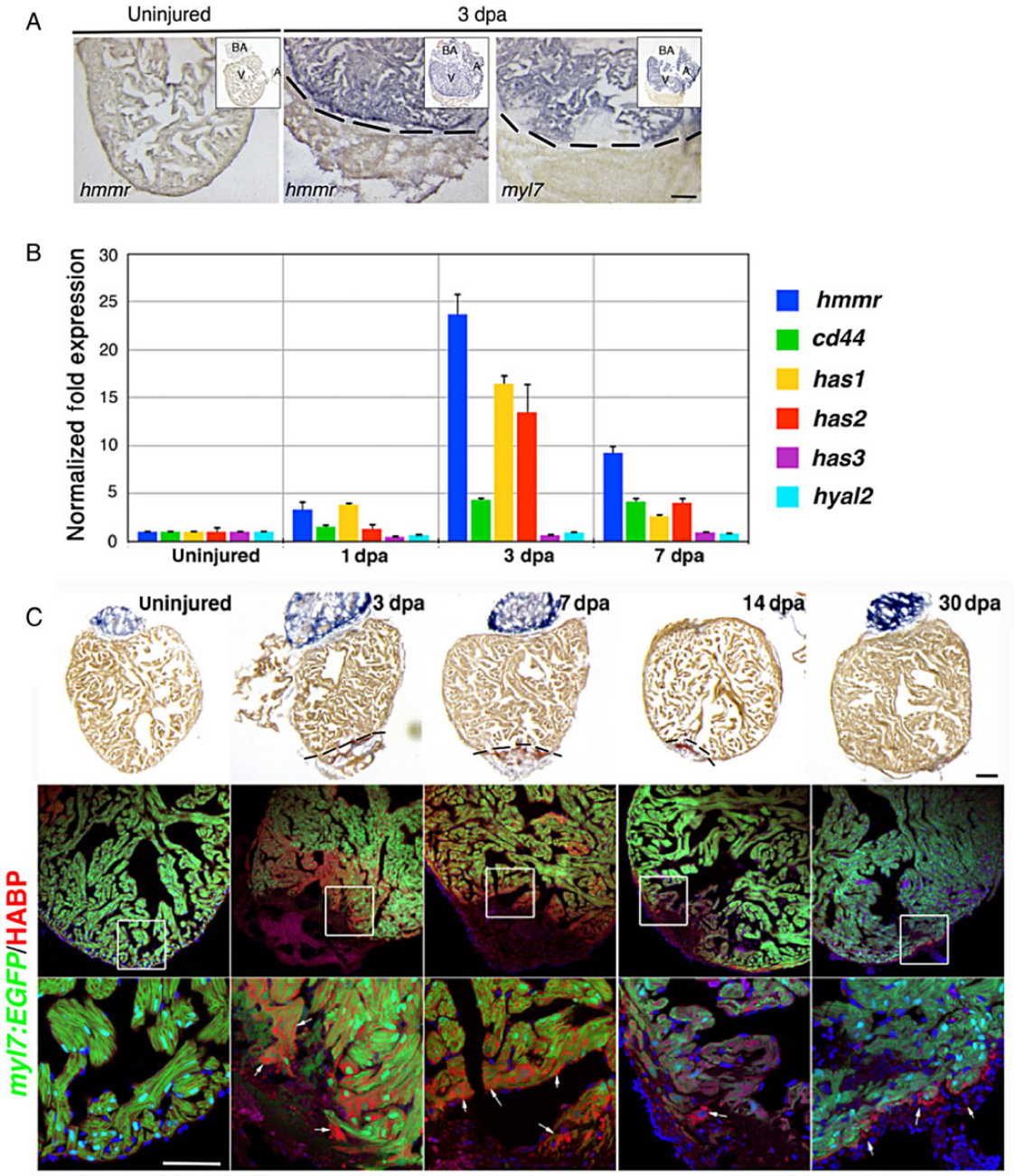

Figure 1 Increased expression of *hmmr, cd44, has-1* and −2, and HA after ventricular resection. (*A*) ISH in hearts showing that *hmmr* is highly expressed at 3 dpa ($n = 5$), compared with the uninjured hearts ($n = 5$). *myl7* labels myocardium. A, Atrium; V, Ventricle; BA, *Bulbus Arteriosus*. Black dashed lines delimitate the injured area. (*B*) Q-PCR analyses of *hmmr, cd44, hyaluronan synthase 1* (*has 1*), 2, and *3*, and *hyaluronidases 2* (*hyal2*) expression at 1, 3, and 7 dpa. Values were normalized to *α-actin* and *RNAP* expression. (*C*) AFOG staining of uninjured hearts ($n = 6$) and hearts at 3 ($n = 13$), 7 ($n = 11$), 14 ($n = 5$), and 30 dpa ($n = 5$). AFOG stains intact cardiac muscle in yellow-orange, fibrin in red, and collagen in blue. Confocal images of *Tg(myl7:EGFP)* hearts showing intact cardiac muscle in green and accumulation of HA after injury in red. HA, detected using biotinylated HABP (bHABP), was found in proximity of the clot (arrows). White boxes show the area where the higher magnification picture was taken. Sections were counterstained with DAPI (blue). Scale bars, 100 μm.

3.4 Inhibition of Has and Hmmr blocks epicardial EMT and their migration

Previous studies have demonstrated the importance of epicardial cell activation and migration into the clot to support angiogenesis.[14] We observed a marked reduction in vessels structures in the regenerating ventricle after HMC or *hmmrVMO* injections, suggesting a defective epicardial response after injury. The lack of coronary vasculature could have arisen from a failure of either activation of epicardial cells or their subsequent EMT and migration into the clot tissue. We determined that HA was localized within the epicardium implicating a role for HA in this tissue (see Supplementary material online, *Figure S10A*).

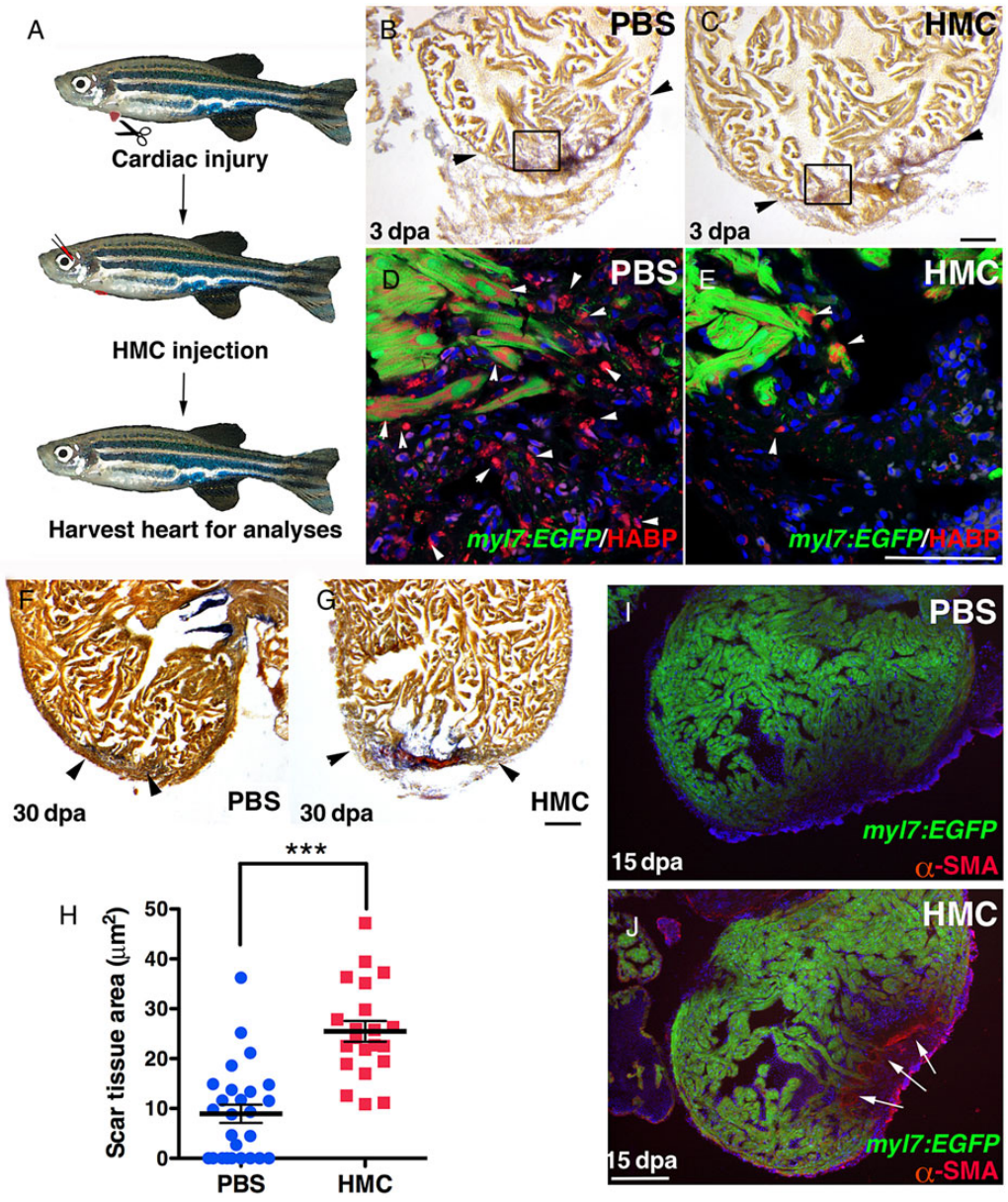

Figure 2 Suppressing HA production blocked cardiac regeneration. (*A*) Experimental outline. Amputation of ventricle apex was performed at Day 0. At 1 dpa, zebrafish were retro-orbital injected daily to deliver HMC or PBS. (*B–E*) HMC injections decreased HA levels in the wound. AFOG staining of *Tg(myl7:EGFP)* hearts at 3 dpa injected with PBS ($n = 7$) (*B*) or with HMC ($n = 7$) (*C*). Black boxes outline area of higher magnification images. Black arrowheads mark scar tissue. (*D*) HA (red) (white arrowheads) was detected within the injury site in control PBS injected, but was markedly diminished after HMC injection (*E*). (*F* and *G*) Hearts at 30 dpa had minimal or no scar tissue in PBS controls ($n = 26$) (*F*), but after HMC injections ($n = 21$) significant scar tissue was observed (*G*). Black arrowheads mark scar tissue. (*H*) Graph of measured scar tissue at 30 dpa in PBS and HMC-injected zebrafish. Black bars indicate the mean and error bars the SEM. (*J* and *I*) αSMA staining for myofibroblasts in *Tg(myl7:EGFP)* hearts at 15 dpa, injected for 6 days with PBS (*I*; $n = 4$) or HMC (*J*; $n = 5$). Sections were counterstained with DAPI (blue). White arrows indicate α-SMA staining. Scale bars, 100 μm. ***$P < 0.001$. Student's *t*-test.

Previous work has shown that HA can stabilize Integrin signalling through phosphorylation of FAK, thus facilitating proper cell migration.[38] FAK is a non-receptor cytosolic tyrosine kinase that is present at the sites of contact between cells and the ECM.[39] In epicardial cells that have migrated into the clot, pFAK (Y-397) was detected in a punctate pattern (see Supplementary material online, *Figure S10B*). To test whether HA is required for epicardial cell activation and migration, HMC was injected into *Tg(wt1b:EGFP)* zebrafish after ventricular resection. Although epicardial Wt1b:EGFP$^+$ cells activation was similar between PBS and HMC-injected fish (see Supplementary material online, *Figure S10C* and *D*), we observed diminished number of EGFP$^+$ cells inside the clot (*Figure 5A–E*) in HMC-injected hearts. HMC treatment also reduced pFAK levels in epicardial cells (see Supplementary material online, *Figure S10E*). Moreover, in PBS-injected hearts, Wt1b:EGFP$^+$ cells appeared more round in shape rather than elongated, suggesting by morphology that epicardial cells are undergoing

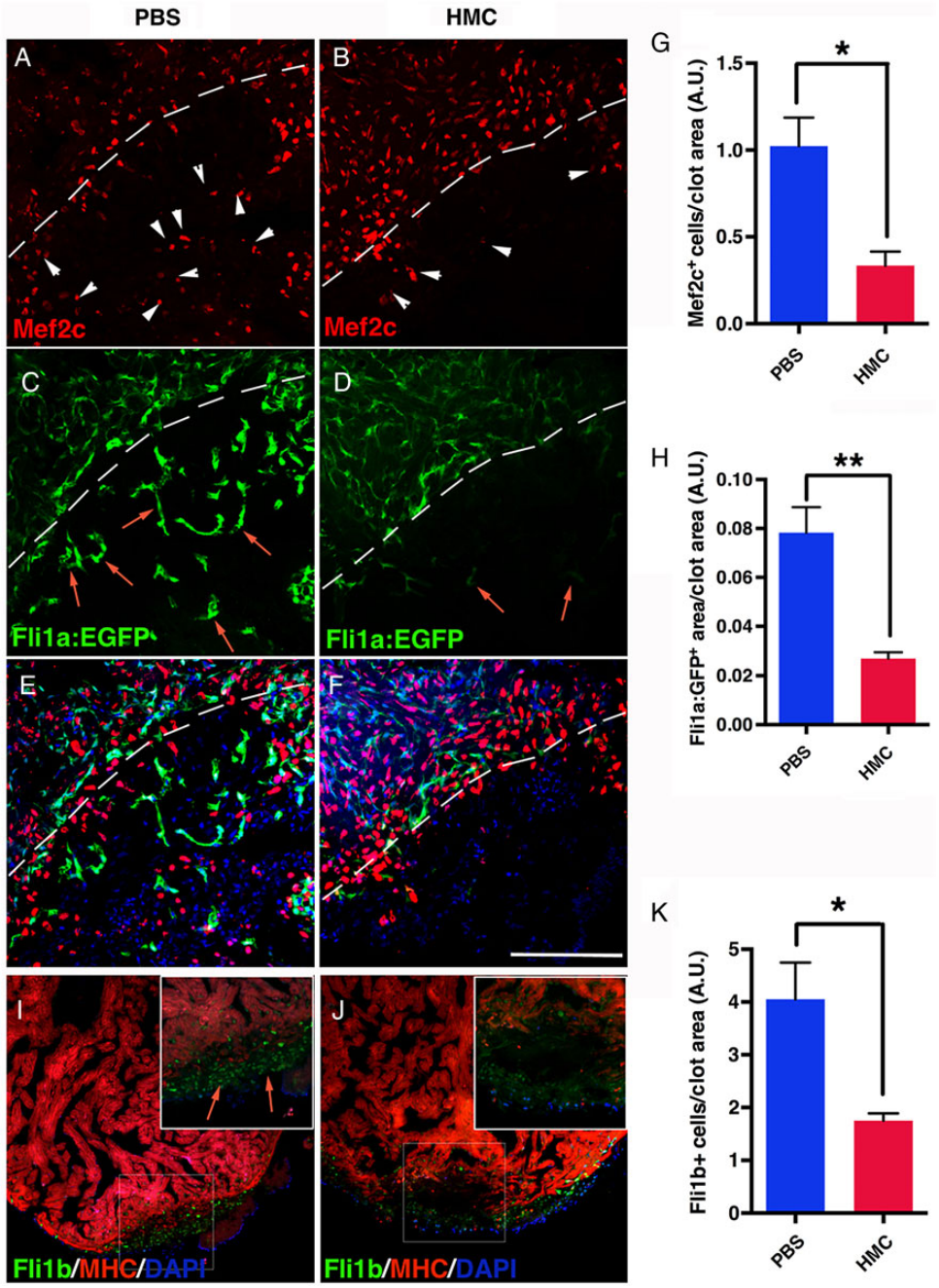

Figure 3 Has inhibition suppressed angiogenesis in regenerating hearts. (*A*) *Tg(fli1a:EGFP)* hearts at 10 dpa, injected for 6 days with PBS ($n = 9$) and (*B*) HMC ($n = 5$). Mef2c immunostaining revealed the presence of cardiomyocytes (white arrowheads) that have populated into the clot area in PBS controls, (*A*), but was clearly decreased after HMC treatment (*B*). White dashed lines delineate the resection plane. The formation of coronary vasculature at 10 dpa was observed through endothelial EGFP expression (red arrows) in *Tg(fli1a:EGFP)* hearts (*C*). In HMC-treated zebrafish (*D*), the presence of EGFP$^+$ cells was reduced. (*E* and *F*) Merged images of *A* and *C*, *B* and *D*. DAPI is shown in blue. (*G*) Graph showing cardiomyocyte number in the regenerating area. (*H*) Quantification of angiogenesis after injection of HMC and PBS, expressed as new vessel area inside the clot. (*I* and *J*) Hearts at 10 dpa, injected for 6 days with PBS (*I*; $n = 5$) or HMC (*J*; $n = 5$) stained for Fli1b expression and MHC. (*K*) Quantification of Fli1b$^+$ cells per unit clot area. Black bars in graphs indicate the mean and error bars the SEM. Scale bars, 100 μm. *$P < 0.05$; **$P < 0.01$. Student's *t*-test.

EMT (Compare *Figure 5F–G* and see Supplementary material online, *Figure S10F*). To confirm that epicardial EMT was suppressed after HMC injections, we performed Q-PCR to measure expression of EMT genes.[16] At 3 dpa, HMC injections reduced the expression levels of *snail1a*, *snail1b*, *snail2*, and *twist1a* (*Figure 5H* and *I*), supporting the notion that HA is required for epicardial EMT. These findings were confirmed with knockdown of Hmmr as the presence of epicardial pFAK was reduced (*Figure 5J–L*). Our studies suggest a role where HA and its receptor Hmmr are required for proper epicardial cell EMT and migration into the wound.

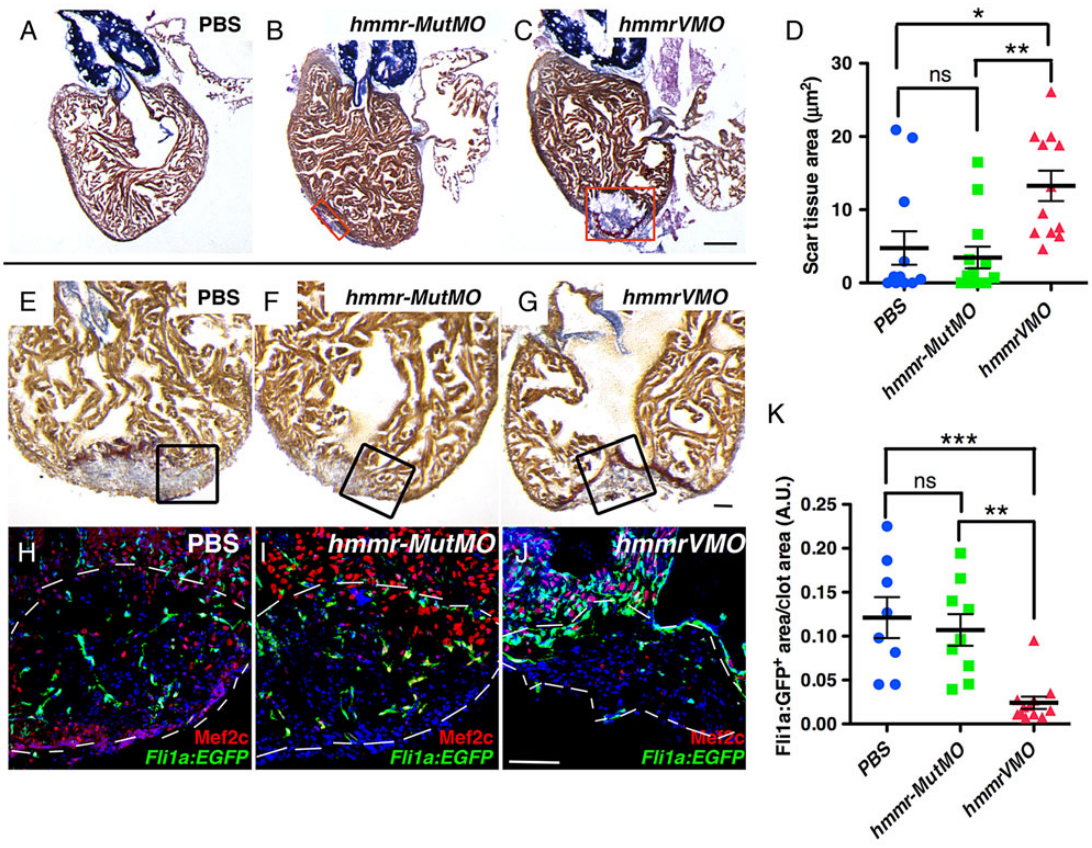

Figure 4 Cardiac regeneration is inhibited after Hmmr knockdown. (*A–C*) AFOG staining of hearts at 30 dpa injected for 10 days with PBS ($n = 12$) (*A*), 5 bp mismatch *VMO* (*hmmr-MutMO*) ($n = 13$) (*B*), and *hmmrVMO* ($n = 12$) (*C*). Red boxes indicate the scar tissue area. Zebrafish injected with *hmmrVMO* contained extensive scar tissue at 30 dpa. (*D*) Graph depicting clot area at 30 dpa. (*E–J*) Hearts at 10 dpa showing clot by AFOG staining (*E–G*), and cardiomyocytes (Mef2c) and coronary vasculature (Fli1a:EGFP$^+$) (H–J). Injection of *hmmrVMO* resulted in decreased Fli1a:EGFP$^+$ cells into the injury site (*J*; $n = 8$), compared with controls (*H*; $n = 16$ and *I*; $n = 6$). (*K*) Quantification of Fli1a:EGFP$^+$ cells in the clot area at 10 dpa after Hmmr knockdown. Black boxes indicate the area where the confocal picture was taken. White dashed lines delimitate the injured area. DAPI is shown in blue. Black bars in graphs indicate the mean and error bars the SEM. Scale bars, 100 μm. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ ns, not significant. One-way ANOVA.

3.5 HA signals through FAK and Src for epicardial EMT

We next addressed the potential role for FAK and Src in heart regeneration as downstream effectors of HA/Hmmr. FAK is autoactivated through tyrosine autophosphorylation at the Y397 site by Integrin/Hmmr interaction and is known to be a critical regulator of cardiac growth and remodelling.[40] This pY397 creates a high-affinity binding site for the SH2 domain of Src family kinases that leads to further phosphorylation of FAK by Src.[41] The activation of FAK/Src complex then regulates downstream signalling pathways that control cell proliferation, spreading, motility, and EMT.[42] In this study, we used chemical inhibition of FAK with PF-573228[43] and the Src kinase inhibitor (SKI-1) to suppress Src,[44] to dissect the role of these proteins in heart regeneration. Inhibiting either FAK or Src suppressed heart regeneration (*Figure 6A–D* and see Supplementary material online, *Figure S11A* and *B*). As observed with blocking HA production, the formation of new vessels was also significantly reduced after injection of PF-573228 or SKI-1 (*Figure 6E–N* and see Supplementary material online, *Figure S11C–F*), and the migration of epicardial cells inside the clot was reduced after SKI-1 injection (see Supplementary material online, *Figure S11G* and *H*), suggesting that Hmmr activation of pFAK and Src is important for epicardial cell function.

In support of these findings, we employed an *ex vivo* assay to monitor epicardial cell migration from intact hearts grown in culture.[28] Decreased epicardial cell spreading from the heart on fibrin-coated wells after *hmmrVMO* injections was observed (*Figure 7A*, *B*, and *F*). Moreover, suppressing FAK (*Figure 7C*, *D*, and G), HA production (*Figure 7E*), or Src activity (*Figure 7H*) also reduced epicardial cell spreading. Taken together, our results implicate a function for HA and Hmmr in directing activated epicardial migration through FAK and Src kinases.

3.6 Accumulation of HA and HMMR is conserved in a mammalian model of MI

In contrast to zebrafish, after MI, adult mammals fail to regenerate injured myocardial tissue. One key difference between these animals is that in mammals cardiomyocytes are post-mitotic, often existing as binuclear or polyploid cells.[45] However, there are some similarities with respect to how the epicardium responds to injury.[46] HA has been reported to accumulate after injury in many tissues and in different species,[30,47] but the presence and function of HMMR remain

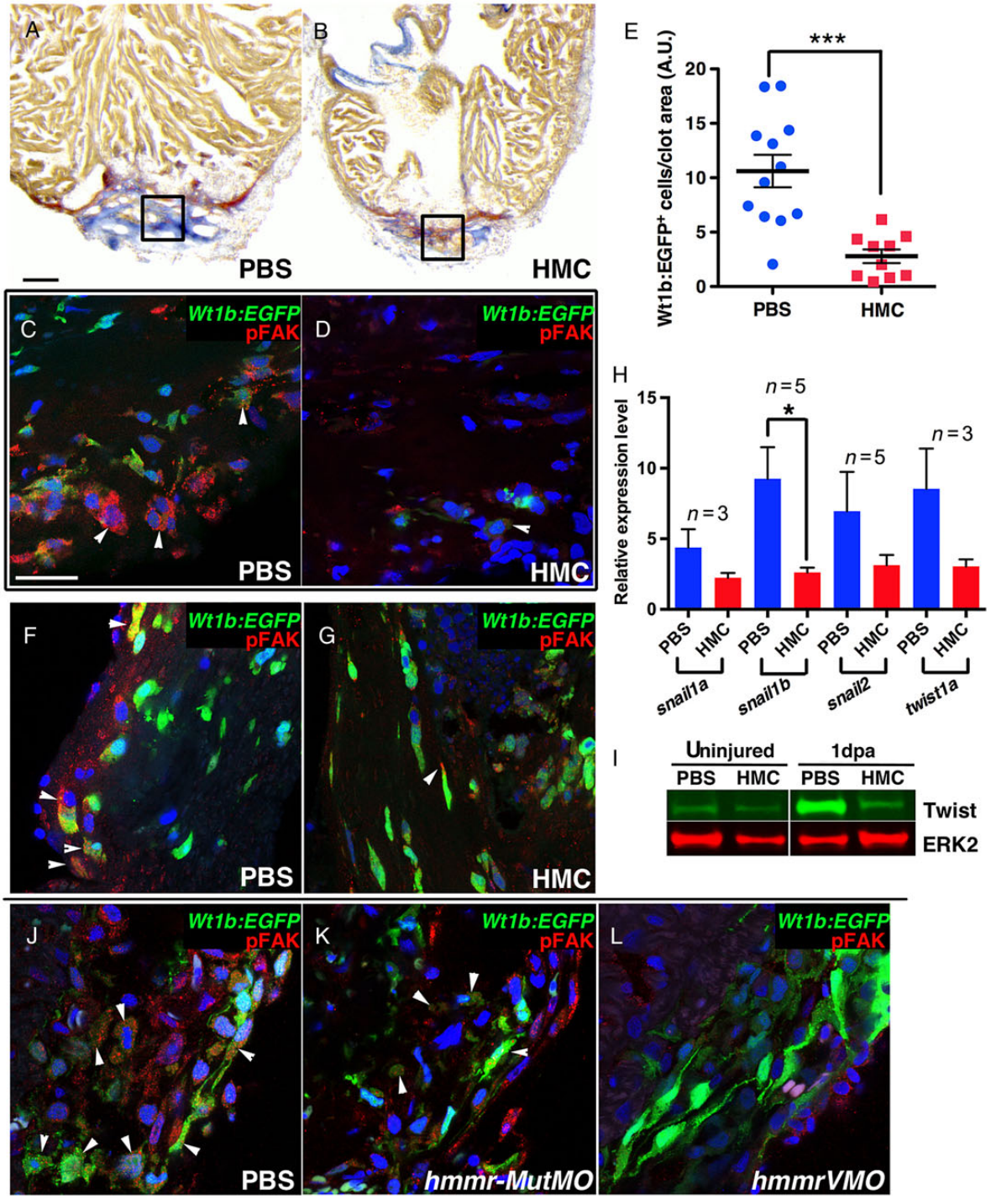

Figure 5 HA is required for epicardial cell EMT. (*A–D*) *Tg(wt1b:EGFP)* zebrafish hearts at 5 dpa injected with PBS ($n = 8$) (*A–C*) or HMC ($n = 6$) (*B–D*) had similar clot area, as shown by AFOG staining (*A* and *B*), but the number of Wt1b:EGFP^{+} cells in the clot was greatly reduced after HMC injections (*D*), compared with PBS (*C*). Black boxes demarcate area of higher magnification. (*E*) Graph showing the number of Wt1b:EGFP^{+} cells in the clot at 7 dpa. (*F* and *G*) Confocal images of activated wt1b^{+} cells at the epicardial region at 3 dpa after injection of control PBS (*F*; $n = 4$) or HMC (*G*; $n = 5$). In controls (*F*), some Wt1b:EGFP^{+} cells were pFAK positive (white arrowhead) and appeared round in shape. In contrast, Wt1b:EGFP^{+} cells after HMC injections (*G*) remained elongated. (*H*) Q-PCR analysis of *snail1a*, *−1b*, *−2*, and *twist1a* expression in ventricular tissue at 3 dpa after injections of HMC. HMC reduced the expression of EMT genes. *n* indicates the number of independent replicates performed for each gene. (*I*) Western blot analysis of Twist1 expression in uninjured hearts and hearts at 1 dpa. Increased Twist1 expression was detected in resected hearts, but was suppressed after HMC injections. Total Erk2 was used as loading control. (*J–L*) *Tg(wt1b:EGFP)* heart at 3 dpa immunostained for pFAK after injection of PBS (*J*; $n = 4$), control *mutant MO* (*K*; $n = 4$), or *hmmrVMO* (*L*; $n = 3$). *hmmrVMO* decreased the number of round Wt1b:EGFP^{+} cells and the number of cells expressing pFAK (*L*). Black bars in graphs indicate the mean and error bars the SEM. Scale bars, 100 μm (*A* and *B*), 25 μm (*C, D, F, G, J, K*, and *L*). *$P < 0.05$; ***$P < 0.001$. Student's *t*-test.

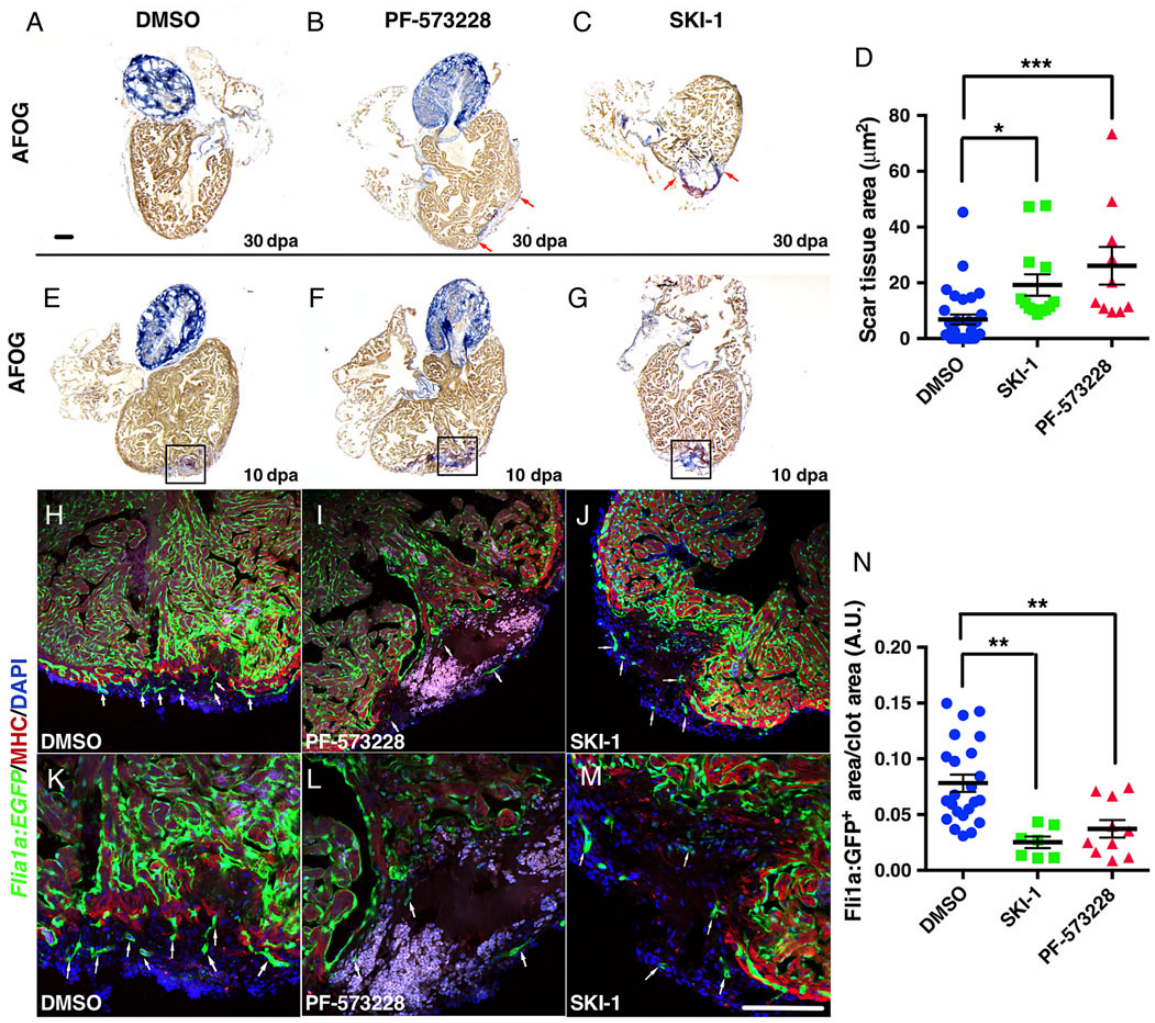

Figure 6 FAK and Src inhibition suppressed angiogenesis in regenerating hearts. (*A*–*C*) At 30 dpa, hearts injected for 10 days with PF-573228, a FAK inhibitor (*B*) ($n = 10$), or with SKI-1 (*C*) ($n = 13$), a Src inhibitor, failed to properly regenerate the heart compared with DMSO controls (*A*) ($n = 31$). Red arrows delineate scar. (*D*) Graph showing clot area at 30 dpa after injections of PF-573228 or SKI-1. (*E*–*M*) PF-573228 and SKI-1 injections inhibited angiogenesis. (*E*–*G*) AFOG staining of hearts at 10 dpa, injected with DMSO (*E*) ($n = 15$), PF-573228 (*F*) ($n = 10$), or SKI-1 (*G*) ($n = 10$). (*H*–*M*) *Tg(fli1a:EGFP)* hearts injected with DMSO (*H* and *K*), PF-573228 (*I* and *L*), or with SKI-1 (*J* and *M*), immunostained for MHC to detect resection plane. White arrows indicate new vessels formed inside the clot. (*N*) Quantification *fli1a*$^{+}$*:EGFP* area in the clot at 10 dpa. FAK and Src inhibition significantly reduced new vessel formation. Black boxes delimitate the areas of confocal imaging. Black bars in graphs indicate the mean and error bars the SEM. Scale bars, 100 μm. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ one-way ANOVA.

understudied in injured mammalian hearts. In uninjured rat hearts, neither HMMR, nor HA was detected (see Supplementary material online, *Figure S12A*, *D* and *G*). However, after MI, HMMR and HA were detected in the scar tissue at 7 days post-MI (dpMI) (see Supplementary material online, *Figure S12B*, *E* and *H*) and at 8 weeks post-MI (wpMI), but not in the myocardium (see Supplementary material online, *Figure S12C*, *F* and *I*). These data support the notion that there is an evolutionary conserved response to cardiac damage with the production of HA and induction of its receptor in scar tissue.

4. Discussion

Using a proteomic approach, we identified increased expression of Atp5a1, desmuslin, and Hmmr in the zebrafish regenerating heart at 3 dpa. Atp5a1 is a critical enzyme in the energetic pathways of cells, and its induction can be interpreted as increasing of energy demand of the damaged heart. This is consistent with results in rat ischaemic myocardium where ATP synthase expression was increased.[48] *Desmuslin* is an intermediate filament, important for the structure of the cell. During embryogenesis, mutation of *Desmuslin* correlates with the development of cardiomyopathies.[49] *Desmuslin* could play a role in the activation and replacement of contractile apparatus, maintaining the cell integrity and restoring the damaged cells in the injured area.

Hmmr is a cell surface receptor for HA, and its main function is to promote cell motility in wound healing.[50] HMMR is particularly interesting because it is detected on the plasma membrane as well as in the cytoplasm and in the nucleus with distinct extracellular and intracellular functions.[51] On the cell surface, HMMR promotes PDGF receptor aggregation to generate sufficient signalling for tissue remodelling.[52] In *Xenopus laevis* tail regeneration studies, HA, *has2*, and *cd44* are

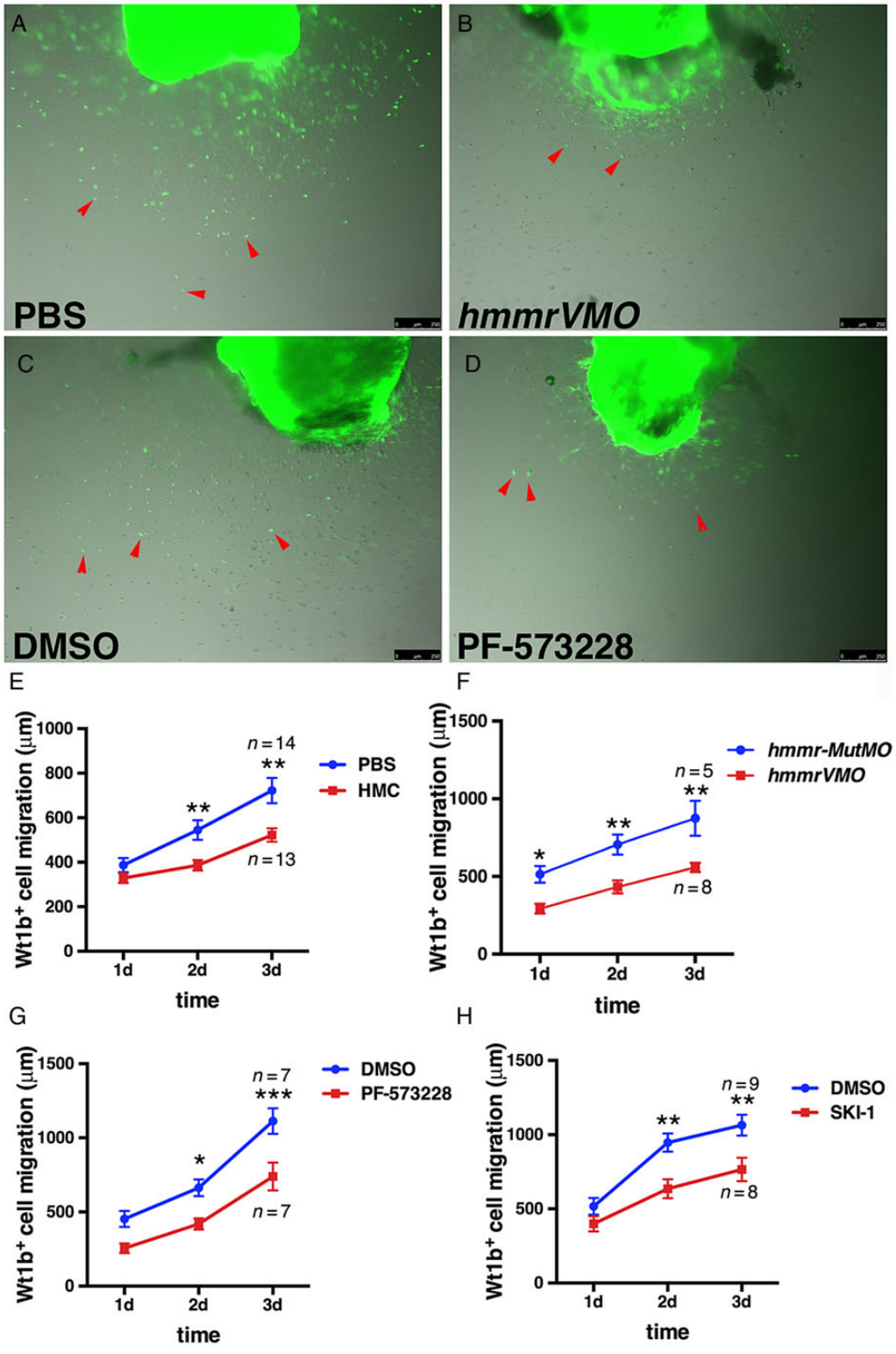

Figure 7 HA and its receptor Hmmr are necessary for epicardial cell migration in *ex vivo* culture. (*A*–*D*) *Ex vivo* epicardial migration assay showing decreased Wt1b:EGFP^{+} migratory behaviour after *hmmrVMO* (*B*) ($n = 8$) and PF-573228 (*D*) ($n = 7$) treatments compared with controls (*A*) ($n = 14$) and (*C*) ($n = 7$). Images were captured at 3 days post-extraction of the heart. The migration of Wt1b:EGFP^{+} epicardial cells (red arrows) was measured from Day 1 to Day 3. In control PBS, DMSO, or *hmmr-MutMO* treatments, epicardial cells migrated on the fibrin-coated plates. In contrast, treatment with *hmmrVMO*, PF-573228 or SKI-1 significantly suppressed epicardial cell migration. (*E*–*H*) Graphs showing migration of Wt1b:EGFP^{+} cells under various treatments, including suppressing HA production in the hearts with HMC (*E*) ($n = 13$), *hmmrVMO* (*F*) ($n = 8$), PF-573228 (*G*) ($n = 7$), and with SKI-1, the Src kinase inhibitor (*H*) ($n = 8$). Black bars in graphs indicate the mean and error bars the SEM. Scale bars, 250 μm. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Two-way ANOVA.

important for mesenchymal cell proliferation.[30] While studies in heart development point to an essential role for Has2 in endocardial cushion, cardiac valve, and epicardium formation,[53–55] as *Has2* knockdown mice die during mid-gestion (E9.5–E10) due to severe cardiac and vascular deformations.[53] In zebrafish, *has2* is strongly expressed in cardiac progenitor cells and is necessary for cardiomyocyte migration and cardiac cone rotation.[29,56,57] However, the exact role for HA and Hmmr in cardiac regeneration has not been explored. Consistent with studies in newts,[58] we report that HA accumulates in adult zebrafish and rat hearts following injury. In this study, we focused on the potential role of HA and Hmmr in regulating cellular migration and proliferation. We observed that decreased production of HA reduced epicardial cell EMT and their migration into the injured area, which is an important step to support coronary vasculature formation. These observations are consistent with studies showing a role for HA to induce EMT through either TGF-β1 or EGF in lung and breast cancer cells.[59] It is also noteworthy that HA can modulate the crosstalk between TGF-β1, PDGF-BB, and CD44 signalling, suggesting that HA is a key player in regulating signalling through these pathways.[60] In zebrafish heart regeneration, PDGF-BB signalling is required for cardiomyocyte proliferation.[11] More recent studies highlight the importance of *pdgfrβ* in epicardial proliferation and EMT.[16] Our studies could point to a potential role for HA and Hmmr in regulating PDGF signalling in epicardial cell EMT. This is further supported with observations that chemical inhibition of FAK activity also resulted in diminished epicardial cell migration. PDGF has been shown to directly activate FAK phosphorylation in lipids rafts.[61] Given that PDGF induced specific FAK tyrosine phosphorylation at position 397 is known to activate Src,[38,61] we also observed a similar lack of epicardial cell migration in zebrafish treated with a Src Kinase Inhibitor. Taken together, these studies suggest that HA and Hmmr play a critical role in epicardial cell EMT after injury through FAK and Src signalling. Our data imply that HA is not only a component of the ECM, but could function as an instructive molecule with PDGF to promote epicardial EMT. Future studies using tissue and cell specific gene knockout of components in this pathway will determine their exact function in epicardial cell EMT and zebrafish heart regeneration.

The relevance of these findings to mammals is supported by the observation that expression of HMMR and HA was induced in rat hearts following ischaemic injury. Other studies have shown that application of HA-based hydrogels into rat hearts after MI can induce neovascularization, reduce infarct area, and more important, improve cardiac function.[62,63] These findings open the possibility of applying HA in mammals to improve cardiac repair after ischaemic injury. Since HA can be present in different sizes and given that the functions of HA can vary according to its molecular weight (MW),[64] it would be of interest to determine whether low or high MW-HA is deposited in the zebrafish ventricle after injury. In conclusion, our study highlights the importance of HA and *hmmr* in epicardial EMT in the regenerating zebrafish heart.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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