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MEKK3 initiates TGFβ2-dependent EMT during endocardial cushion morphogenesis

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Abstract

Congenital heart defects (CHDs) occur at a rate of five percent and are the most prevalent birth defects. A better understanding of the complex signaling networks regulating heart development is necessary to improve repair strategies for CHDs. The MAP3 kinase, MEKK3, is important to early embryogenesis, but developmental processes affected by MEKK3 during heart morphogenesis have not been fully examined. We identify MEKK3 as a critical signaling molecule during endocardial cushion development. We report the detection of MEKK3 transcripts to embryonic hearts prior, during and after cardiac cushion cells have executed epithelial to mesenchymal transformation (EMT). MEKK3 is observed to endocardial cells of the cardiac cushions with a diminishing gradient of expression into the cushions. These observations suggest that MEKK3 may function during production of cushion mesenchyme as required for valvular development and septation of the heart. We used a kinase inactive form of MEKK3 ($MEKK3^{KI}$) in an in vitro assay that recapitulates in vivo EMT, and show that $MEKK3^{KI}$ attenuates mesenchyme formation. Conversely, constitutively active MEKK3 (ca-MEKK3) triggers mesenchyme production in ventricular endocardium, a tissue that does not normally undergo EMT. MEKK3-driven mesenchyme production is further substantiated by increased expression of EMT-relevant genes including TGFβ2, Has2, and periostin. Furthermore, we show that MEKK3 stimulates EMT via a TGFβ2-dependent mechanism. Thus, the activity of MEKK3 is sufficient for developmental EMT in the heart. This knowledge provides a basis to understand how MEKK3 integrates signaling cascades activating endocardial cushion EMT.

Keywords

epithelial-to-mesenchymal transition; MEKK3; TGFβ2; endocardial cushions; heart

Introduction

The formation of endocardial cushions is an important step in valvuloseptal development. After the heart has undergone rightward looping, extracellular matrix (ECM) deposits in the atrioventricular canal (AVC) and outflow tract (OFT) form the initial endocardial cushions[1].

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None.

A vital event in endocardial cushion morphogenesis is epithelial to mesenchymal transition (EMT), where specific cells in the endocardium surrounding the cardiac cushions are activated to delaminate from the endocardial layer, transform into mesenchyme, and migrate into the underlying ECM[2]. This newly formed tissue will be remodeled to form valves and septa creating the partitioned four-chamber heart.

Approximately five percent of children born have a congenital heart defect (CHD)[3]. CHDs include valvular and septal defects that cause insufficient blood flow through the heart. These patients often require surgery during infancy and such defects can cause complications later in life. Although there is increased knowledge about genetic factors that are responsible for specific CHDs, little is known how these genetic errors translate into CHD phenotypes. Misexpression or improper coding of effector proteins may lead to defective extracellular and/or intracellular signaling, resulting in altered cellular processes critical to heart development. Marfan's syndrome is an example of disrupted signaling during heart formation. A mutation in the Fibrillin-1 gene, an extracellular matrix protein that regulates TGFβ, results in myxomatous valves[4]. There are also a variety of Marfan-like diseases where mutations in the TGFβ receptors are responsible for valvular defects[5], further suggesting that TGFβ signaling must be well-regulated during valvulogenesis. Another example is Noonan's syndrome, a disorder caused by dysregulated signal transduction by an overactive tyrosine phosphatase, Shp2[6]. Transgenic mice with a common Shp2 mutation, Q79R, found in Noonan's patients are prone to pulmonary valve stenosis due to elevated cell proliferation and reduced apoptosis during endocardial cushion development[7, 8]. Wild-type Shp2 activity is involved in MAPK signaling downstream of receptor tyrosine kinases[9], such as Epidermal Growth Factor Receptor (EGFR), which regulate heart valve formation[10]. Regulation of MAPK pathways, including Ras-Raf-MEK-ERK, is affected by TGFβ[11], but this has yet to be examined in endocardial cushion development.

Among signaling factors important for cardiac cushion EMT are BMP2 and TGF-β2. BMP2 is vital for development of endocardial cushions as conditionally removing BMP2 from AVC myocardium causes abnormal segmentation of AV myocardium and failure to form endocardial cushions[12]. TGFβ2 is also important in activating endocardial cushion EMT and regulating cushion morphogenesis[13–15]. Mice deficient for TGFβ2 exhibit cardiac defects including dilated aortic walls, overriding tricuspid valves, myxomatous valves, and outflow tract malformations[15, 16]. Although TGFβ family growth factors are important to cardiac cushion development, the regulation and action of these factors is not completely understood.

MAPK cascades often begin with ligand-mediated activation of a receptor. Those involved in developmental processes include receptor tyrosine kinases, G-protein coupled receptors, and TGFβ/BMP receptors among others. The activated receptor recruits intracellular effectors that mediate specific signaling pathways. For example EGFR receptor tyrosine kinase activation leads to recruitment of effectors, such as Grb2 and Sos, which lead to activation of the small GTPase, Ras. Consequently, a MAP3K, like Raf, will be activated by Ras, so that it phosphorylates and activates downstream MAPKK (MEK). The activated MAPKK will then phosphorylate a MAPK (p38, ERK, ERK5, or JNK), which translocates into the nucleus to mediate the activities of transcription factors[9, 17]. These cascades are responsible for control of cell proliferation, differentiation, apoptosis, or migration. MAPKs, such as ERK, have been implicated during endocardial cushion development[7, 8, 18], but their roles are still being defined. Regulators of ERK MAPK, including Shp2, Ras, and Raf, are also implicated in proper heart valve development as mutations in these genes affect cardiac cushion EMT and cause valvular defects[7, 19–21].

We have previously shown that the MAPKKK, MEKK4, is necessary but not sufficient for cardiac cushion EMT during heart development[22]. The current work elucidates the role of

MEKK3 with different functions than MEKK4 during cardiac cushion morphogenesis. Our results show that MEKK3 is both necessary and sufficient for EMT in the embryonic heart further supported by previous results that demonstrate loss of MEKK3 is embryonic lethal[23]. Finally, MEKK3 functions via a TGFβ2-dependent mechanism to produce valve mesenchyme.

Materials and Methods

Immunodetection on embryo sections

Mouse embryos were collected from stages E9.5 to E12.5 and processed as described previously[22]. Rabbit polyclonal antibody[24] and goat-anti-rabbit-Alexa594 secondary antibody (Molecular Probes, Invitrogen, Carlsbad, CA) was used to detect MEKK3 documenting fluorescence with a Leica DMLB fluorescence microscope (Leica, Bannockburn, IL) with Image ProPlus software (Media Cybernetics, Bethesda, MD). For vimentin detection, an anti-vimentin antibody (Santa Cruz Biotechnology, Santa Cruz, CA)[22] was used with an anti-goat-Alexa594 secondary antibody (Molecular Probes). Nuclear staining was accomplished with Hoechst dye (Molecular Probes). Tissue sections and cells treated with only secondary antibody were used to control for background staining (Not shown).

RT-PCR and Real-time RT-PCR

RNA was isolated from embryonic hearts at stages E9, E9.5, E10.5 and E14.5 with Trizol (Invitrogen). Reverse transcription reactions were accomplished using a reverse transcription reaction kit (Fermentas, Glen Burnie, MD). Primers for PCR are described in supplemental methods.

Real-time PCR was performed using a Roche 480 LightCycler (Roche, Indianapolis, IN). Real-time RT-PCR was done in triplicate and statistics are included as standard deviation.

endocardial cushion and ventricular explants

Embryos were removed from pregnant female mice at E9.5 (Sigma, St Louis, MO). Hearts were dissected from embryos at E9.5 and microdissection of the AVC cushions and ventricles was performed in Tyrode's Buffer[14, 19, 25]. AVCs and ventricles were dissected and placed in adenoviral-transfection mixes as previously described[22]. Four explants were placed per type I collagen gel and hydrated overnight with 0.1% ITS in Opti-MEM (Gibco-BRL) according to published methods[14, 22]. Additional transfection mix was placed on each explant for 3 hours and replaced by complete media (1% BGS (Bovine Growth Serum, Hyclone, Logan, UT), 1% Anti-Mycotic/Anti-Biotic, 1X M199 (Gibco-BRL), 0.01% ITS (Gibco-BRL)). AVC explants were allowed to incubate at 37°C for 48 hours, while ventricular explants incubated at 37°C for 72 hours. Cultures were used for RNA isolation or fixed with 2% paraformaldehyde for detection of vimentin and cell enumeration. For TGFβ2 neutralizing experiments, anti-TGFβ2 and IgG-isotype control (R&D systems, Minneapolis, MN) were used at a concentration of 250 pg/ml in media[26]. Other neutralization treatments are described in supplemental methods. Some cultures were provided with BrdU as detailed in the supplemental section. Statistics for experiments were calculated using the Student's two sample unpaired T-test and standard deviation.

Preparation of adenoviral-mediated transfection mix for explants

Adenoviral-mediated transfections were accomplished as previously described[19, 22]. Plasmids for mammalian expression (pCMV) containing wt-MEKK3, the kinase domain of MEKK3 (ca-MEKK3), or MEKK3^{KI} (K391M) (1 μg per explant) were added with adenovirus that expresses GFP (Ad5-GFP, 10^8 pfu) (Iowa Gene Vector Transfer Core) in Opti-MEM (Gibco-BRL). Details are listed in online supplement.

Results

MEKK3 expression in the embryonic heart

MEKK3 transcripts are detected in the rudimentary heart at stages E9, E9.5, E10.5, and E14.5, stages before, during and following cardiac cushion EMT[14] (Online Figure I A). To localize MEKK3 protein in the embryonic heart, we performed immunofluorescence staining on embryo sections from E9.5 to E12.5 with an anti-MEKK3 antibody. MEKK3 is detected to the myocardium at all stages examined. At E9.5 to E10.5, MEKK3 is also detected to the endocardium lining the AVC cushions, and a diminished gradient of expression in mesenchymal cells within the cushion matrix (Figure 1 A, B, D, E). Additionally, little MEKK3 is detected in the ventricular endocardium, which does not normally undergo transition to mesenchyme (Figure 1 C, F). This immuno-localization places MEKK3 in the appropriate area of the heart when EMT occurs. At later stages E11.5 (Online Figure I B, C) and E12.5 (Online Figure I D, E), MEKK3 is localized to the endocardium, although moderately diminished at E12.5. This prolonged expression of MEKK3 beyond the EMT period suggests other roles for MEKK3 in endocardial cell function.

MEKK3 is necessary for cardiac cushion EMT

In vivo EMT is recapitulated in vitro via the endocardial cushion explant assay[2]. In this assay, endocardial cushions of the AVC are microdissected from E9.5 embryos and explanted onto collagen gels[14]. The endocardial cells migrate onto the gel, where they undergo EMT and invade into the collagen matrix. Mesenchyme formation and invasion is scored to determine the extent of EMT. Mesenchymal cells are distinguished as individual, elongated cells with filapodia, whereas epithelial cells have cell-cell contacts with adjacent cells and have a rectangular, or rounded morphology[2, 19]. In addition, immunoflourescent detection of vimentin indicates mesenchymal cells, since its expression is upregulated in cells of this phenotype[27]. We used this assay to determine whether the kinase activity of MEKK3 is required for EMT and production of AVC cushion mesenchyme. Kinase inactive MEKK3 ($MEKK3^{KI}$), which acts as a dominant negative, was used in the AVC endocardial cushion explant assay. $MEKK3^{KI}$ was created by mutating the active site lysine at position 391 to methionine (K391M)[24]. Naïve and Ad5-GFP infected cultures were used as controls and displayed normal amounts of mesenchyme production (Figure 2 A, B, D). Addition of wild-type MEKK3 (wt-MEKK3) to explant cultures did not have an effect on AVC cushion EMT (Figure 3 D). In contrast, $MEKK3^{KI}$ causes a 3-fold reduction in mesenchyme formation compared to control explant cultures (Figure 2 C, D). Active caspase-3 detection in AVC explants with $MEKK3^{KI}$ shows a significant 2-fold increase in apoptosis compared to controls after 24 hours (Figure 3 A, B, C, $p = 0.0077$). This suggests that MEKK3 kinase activity is necessary for developmental EMT during endocardial cushion morphogenesis and functions in endothelial cell survival.

MEKK3 is sufficient to activate EMT

Ventricular endocardium does not undergo EMT because the myocardium of the ventricle is not competent to promote transformation of AVC endocardium, suggesting the myocardium underlying the AVC is regionally restricted for promoting mesenchyme formation[2]. Therefore, explants of endocardium derived from the ventricle are used to screen candidate factors for sufficiency to induce EMT. For example, Alk2, a TGFβ/BMP receptor, has been determined sufficient for EMT in this manner[28]. We established ventricular endocardial explants to determine whether the catalytic activity of MEKK3 can drive EMT. A constitutively active form of MEKK3 (ca-MEKK3) was created by truncating the N-terminal region, leaving the fully-active kinase domain[29]. Naïve and Ad5-GFP transfected ventricular endocardium exhibited little to no mesenchyme formation (Figure 4 A, D). Explants treated with constitutively active Alk2 (ca-Alk2), which has been shown to activate EMT in this

system[28], served as a positive control, driving mesenchyme outgrowth (Figure 4 B, D). ca-MEKK3 also induced EMT in explanted ventricular endocardium to levels comparable to those with ca-Alk2 (Figure 4 B, C, D). Mesenchymal cells were confirmed by vimentin detection in cells of ca-MEKK3 and Alk2 control cultures (Figure 4 B, C). In addition, cell proliferation was examined by BrdU incorporation into ventricular explant cultures. The number of proliferating cells is significantly increased in ca-MEKK3 cultures (Figure 6, $p = 0.0037$). Increased proliferation is observed primarily within endothelial cells (Figure 5, $p = .0007$), suggesting that ca-MEKK3 is primarily involved in endothelial cell proliferation. These data demonstrate that ca-MEKK3 is sufficient for the production of cardiac mesenchyme, partly by increasing the population of endocardial cells available for EMT.

MEKK3 triggers expression of factors that promote EMT

A TGFβ2/TGFβ type III receptor (TβRIII) signaling cascade is critical for the EMT program[30]. Additionally, molecules including BMP2, Has2, and Snail2 (Slug) are implicated in this process[12, 19, 31]. Importantly, periostin is a marker of mature cushion mesenchyme[32, 33]. Therefore, real time RT-PCR was performed on RNA samples prepared from ventricular explants with or without ca-MEKK3 to examine the expression of these EMT-related genes. MEKK3 mRNA is increased in ventricular explants provided with ca-MEKK3, which along with fluorescence detection of GFP (Online Figure II), demonstrates transfection of the cultures (Figure 6). TβRIII is expressed 64 times more in the ca-MEKK3 samples compared to controls (Figure 6). TβRIII is required to mediate TGFβ2- induced EMT[30, 34]. As TGFβ2 is necessary in promoting EMT in endocardial cushions[14, 26], we detect a substantial increase in TGFβ2 message in ca-MEKK3 samples, but not in controls (Figure 6). Furthermore, expression of Snail2 (Slug), a transcription factor up-regulated by TGFβ2 signaling[31], is also induced by ca-MEKK3-expressing ventricular cultures (Figure 6). BMP2 is also necessary for mesenchyme production from the AVC cushion endocardium, and is expressed in AVC cushion myocardium, but not in the ventricular myocardium[12,35]. BMP2 expression is not observed in control ventricular explant cultures, but ca-MEKK3 induces expression of BMP2 in this system (Figure 6). TGFβ1 and TGFβ3 are also induced by ca-MEKK3 as compared to control samples (Figure 6). Examining expression of genes that are normally activated by cells undergoing EMT[19], we detect induced expression of hyaluronan synthase 2 (Has2) compared to controls, futher supporting a role for MEKK3 in mesenchyme production (Figure 6). Periostin expression confirms the mature mesenchymal phenotype of cells that invade into the extracellular matrix[36,32]. Expression of periostin is significantly increased in ca-MEKK3 cultures compared to controls (Figure 6), which coincides with the increase in mesenchymal cells observed in Figure 4. These data suggest that ca-MEKK3 in ventricular explants cultures is inducing expression of genes necessary for the onset of EMT and eventually genes that are indicative of mesenchyme production.

ca-MEKK3 induces production of TGFβ2

The observation that MEKK3 activity increases expression of TGFβ2, TβRIII, and Slug suggests that it may be upstream of this key TGFβ growth factor cascade as required for EMT. Therefore, we examined explant supernatants for the presence of TGFβ2 to determine if there is an increase in TGFβ2 that coincides with elevated gene expression. A significant 4-fold increase in TGFβ2 is detected in ca-MEKK3 explant cultures compared to GFP controls (Figure 7 A). Conversely, a 4.4-fold decrease in TGFβ2 is detected in endocardial cushion explants treated with kinase inactive MEKK3 ($MEKK3^{KI}$) (Online Figure III). Thus, MEKK3 increases production of TGFβ2 message and protein. Next, we examined phosphorylated-Smad2 (p-Smad2) as a functional target of TGFβ signaling in ventricular explants cultures with or without ca-MEKK3. Detection of filamentous actin (F-actin, green) shows maintained cell-cell junctions between ventricular endocardial cells in controls (Figure 7 B), while these junctions are dismantled in the presence of ca-MEKK3 indicating initiation of EMT (Figure 7 C).

Although low levels are detected in control ventricular endocardium, we observe increased p-Smad2 in cultures with ca-MEKK3 (Figure 7, compare D and E). Collectively, these observations support that MEKK3 induces EMT through TGFβ2.

Neutralization of TGFβ2 inhibits ca-MEKK3 induced EMT -

Our observations strongly suggest MEKK3 stimulates TGFβ2 production coincident with EMT. To determine whether TGFβ2 is a central factor mediating MEKK3-induced EMT, ca-MEKK3 expressing ventricular explant cultures were cultured with or without neutralizing anti-TGFβ2 antibody. As expected, ca-MEKK3 is able to induce mesenchyme production in explants treated with control IgG (Figure 8 C) and those with ca-MEKK3 alone (Figure 8 compare A and B). In contrast, addition of anti-TGFβ2 dramatically inhibits the ability of ca-MEKK3 to stimulate mesenchyme formation (Figure 8 D, E). Since increases in expression were also observed for BMP2, TGFβ1, and TGFβ3, it was necessary to examine whether these factors have a significant contribution to ca-MEKK3-induced mesenchyme production. Effects of neutralizing each of these factors in cultures of ventricular endocardium with ca-MEKK3 were assessed for EMT. We used BMP antagonist, noggin, and antibodies against TGFβ3 and TGFβ1 in these experiments. Decreases in EMT are observed with noggin and anti-TGFβ3 treatment in ca-MEKK3 expressing ventricular explants, however, mesenchyme production in each was significantly higher than with anti-TGFβ2 treatment (Figure 8 E, Online Figure III C, D). Blockade of TGFβ1 has no significant effect on ca-MEKK3-induced EMT (Figure 8 E, Online Figure III E). Thus, neutralizing TGFβ2 induced by ca-MEKK3 blocks EMT restoring the normal phenotype of the ventricular endocardium which does not normally undergo EMT. Blocking activities of BMP2, TGFβ3, or TGFβ1 does not fully restore the normal phenotype (Online Figure IV). Furthermore, TGFβ2 is elevated even with blockade of BMP2, TGFβ3, or TGFβ1 compared with control cultures (Online Figure V). The dramatic reduction in EMT when TGFβ2 is neutralized also suggests that the other TGFβ factors do not compensate for TGFβ2 loss in this system. Collectively, these data show that the production of endocardial-derived mesenchyme by active MEKK3 involves a TGFβ2-dependent mechanism.

Discussion

MEKK3 knockout mice exhibit myocardial and endocardial defects, which are in part responsible for their death at E10.5[37]. Although not a primary focus of Yang et al.[37], the authors' show an acellular AVC cushion in the MEKK3-deficient embryos. These defects are consistent with localization of MEKK3 to the myocardium and AVC cushion endocardium in our studies. Importantly, we detect very little MEKK3 in ventricular endocardium, which is a region in the heart that does not execute developmental EMT[2]. This restricted detection of MEKK3 to the cushion endocardium is consistent with expression patterns of other proteins necessary and sufficient for cardiac cushion EMT. One example is Alk2, a TGFβ/BMP receptor, which is sufficient to activate mesenchyme production during valvulogenesis[28]. Hence, localization of MEKK3 to the AVC cushion endocardium implies that it is also involved in activation of endocardium to become mesenchyme. Moreover, MEKK3 kinase activity is necessary for the transition from endocardium to mesenchyme, since endocardial cushion explants faile to display normal levels of mesencymal cells with $MEKK3^{KI}$. We observe endocardial migration onto the gel, but a majority of cells do not become vimentin-positive mesenchymal cells in the presence of $MEKK3^{KI}$ (See Online Figure II and Figure 2). There is also an increase in apoptosis observed in AVC explant cultures provided with $MEKK3^{KI}$, which is consistent with MEKK3 being involved in endothelial cell survival in MEKK3 deficient mice[38]. Remarkably, ca-MEKK3 is sufficient to induce EMT from ventricular endocardium, similar to that with ca-Alk2[22, 28]. Additionally, an increase in endothelial cell proliferation is observed in ca-MEKK3 expressing ventricular endocardium, consistent with decreased endothelial cell proliferation in the MEKK3-deficient embryonic heart [38]. Together,

these data demonstrate that MEKK3 is necessary and sufficient for mesenchyme production and this is partially due to increased proliferation and survival of endocardial cells available for the EMT program.

Our studies show that TGFβ2 is necessary for ca-MEKK3-driven EMT in ventricular endocardium. This growth factor is especially important for mesenchyme production in the murine heart[14]. TGFβ2 involvement in endocardial cushion morphogenesis is supported by a TGFβ2 knockout mouse model that exhibits heart defects including valve and outflow tract malformations[15, 16]. This phenotype also demonstrates that TGFβ2 has developmental functions which are not compensated for by the other TGFβs. In addition, MEKK3 is upstream of the MAPKs, p38 and JNK, which are important in activating ATF2, a transcription factor that stimulates TGFβ2 expression[39]. Normally ventricular endocardium expresses little TβRIII, a receptor necessary for TGFβ2-induced EMT [30]. In our studies, TβRIII and TGFβ2 are both upregulated in response to ca-MEKK3, demonstrating a link between the MAP3K, MEKK3, and a defined EMT-activating pathway. Mesenchyme production by ca-MEKK3 in ventricular endocardium was ablated by neutralizing TGFβ2 demonstrating that MEKK3 is upstream of TGFβ2-mediated EMT. Consistent with this, we detect an increase in Snail2[31] during ca-MEKK3 conditions suggesting intact TGFβ2 activity during MEKK3 initiated EMT. We detect a number of additional genes upregulated as a result of MEKK3 activity including Has2, which is a synthetase that produces hyaluronan a vital extracellular molecule required for cardiac EMT [19]. Another gene upregulated by MEKK3 is TGFβ3, which becomes expressed in cushion mesenchyme, and contributes to formation of fibrous structures in the developing heart [23]. In addition, BMP2 message is induced by ca-MEKK3, but not in control cultures. Alk3, a BMP receptor, in the myocardium is needed for TGFβ2 production and cushion morphogenesis, so MEKK3 may act upstream of BMP2 signaling for TGFβ2-dependent EMT [40]. BMP2 also promotes cell migration and tissue maturation during endocardial cushion morphogenesis demonstrating its additional role in post-EMT events [41]. Interestingly, BMP2 is shown to act synergistically with TGFβ3 to promote cardiac cushion EMT in chicken [42]. Since we show that neutralizing BMP2 or TGFβ3 in ca-MEKK3 expressing ventricular explants does not decrease mesenchyme production to the extent by blocking TGFβ2, it is likely that these molecules either enhance TGFβ2-induced EMT or play a role in the invasion step of EMT. TGFβ1 expression increases with ca-MEKK3 in ventricular explants, however, there is no significant decrease in MEKK3-driven mesenchyme production after blockade of TGFβ1. Embryos from female TGFβ1 knockout mice demonstrate in utero mortality with cardiac defects, whereas females that are heterozygous or wild-type for TGFβ1 are able to transfer TGFβ1 to the developing embryo for normal cardiac development [43]. Letterio et al. also indicate that TGFβ1 is important to endocardial cell survival [43]. This may explain why fewer cells are observed with neutralizing TGFβ1 than other groups (not shown), although the percentage of cells undergoing EMT is relatively equal to ca-MEKK3 expressing ventricles. Periostin is expressed by mesenchymal cells within the endocardial cushions [36], and its overexpression causes transition of cells to mesenchyme [44]. Periostin expression is significantly increased in samples with ca-MEKK3 versus controls, which supports the ability of MEKK3 to produce mature mesenchyme. Collectively, our studies show that MEKK3 mediates EMT and production of cushion mesenchyme via a TGFβ2-dependent mechanism.

This is the first time that MEKK3 has been implicated in developmental EMT and functionally linked to early events in heart valve formation. We observe MEKK3 is mediating pathways, specifically through TGFβ2, that are activating EMT. As such, active MEKK3 is sufficient for mesenchyme production. Deciphering upstream mediators of MEKK3 will be important to connect factors that establish an activated endothelium with the ability to execute EMT. In summary, we present MEKK3 as a critical signaling effector in the pathways that mediate endocardial cushion EMT.

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References

1. Markwald RR, Fitzharris TP, Manasek FJ. Structural development of endocardial cushions. Am J Anat 1977;148:85–119. [PubMed: 842477]
2. Runyan RB, Markwald RR. Invasion of mesenchyme into three-dimensional collagen gels: a regional and temporal analysis of interaction in embryonic heart tissue. Developmental biology 1983;95:108–114. [PubMed: 6825921]
3. Pierpont ME, Basson CT, Benson DW Jr, Gelb BD, Giglia TM, Goldmuntz E, McGee G, Sable CA, Srivastava D, Webb CL. Genetic basis for congenital heart defects: current knowledge: a scientific statement from the American Heart Association Congenital Cardiac Defects Committee, Council on Cardiovascular Disease in the Young: endorsed by the American Academy of Pediatrics. Circulation 2007;115:3015–3038. [PubMed: 17519398]
4. Ng CM, Cheng A, Myers LA, Martinez-Murillo F, Jie C, Bedja D, Gabrielson KL, Hausladen JM, Mecham RP, Judge DP, Dietz HC. TGF-beta-dependent pathogenesis of mitral valve prolapse in a mouse model of Marfan syndrome. J Clin Invest 2004;114:1586–1592. [PubMed: 15546004]
5. Singh KK, Rommel K, Mishra A, Karck M, Haverich A, Schmidtke J, Arslan-Kirchner M. TGFBR1 and TGFBR2 mutations in patients with features of Marfan syndrome and Loeys-Dietz syndrome. Hum Mutat 2006;27:770–777. [PubMed: 16799921]
6. Sznajer Y, Keren B, Baumann C, Pereira S, Alberti C, Elion J, Cave H, Verloes A. The spectrum of cardiac anomalies in Noonan syndrome as a result of mutations in the PTPN11 gene. Pediatrics 2007;119:e1325–e1331. [PubMed: 17515436]
7. Araki T, Mohi MG, Ismat FA, Bronson RT, Williams IR, Kutok JL, Yang W, Pao LI, Gilliland DG, Epstein JA, Neel BG. Mouse model of Noonan syndrome reveals cell type- and gene dosage-dependent effects of Ptpn11 mutation. Nat Med 2004;10:849–857. [PubMed: 15273746]
8. Krenz M, Yutzey KE, Robbins J. Noonan syndrome mutation Q79R in Shp2 increases proliferation of valve primordia mesenchymal cells via extracellular signal-regulated kinase 1/2 signaling. Circ Res 2005;97:813–820. [PubMed: 16166557]
9. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001;2:127–137. [PubMed: 11252954]
10. Chen B, Bronson RT, Klaman LD, Hampton TG, Wang JF, Green PJ, Magnuson T, Douglas PS, Morgan JP, Neel BG. Mice mutant for Egfr and Shp2 have defective cardiac semilunar valvulogenesis. Nat Genet 2000;24:296–299. [PubMed: 10700187]
11. Suzuki K, Wilkes MC, Garamszegi N, Edens M, Leof EB. Transforming growth factor beta signaling via Ras in mesenchymal cells requires p21-activated kinase 2 for extracellular signal-regulated kinase-dependent transcriptional responses. Cancer Res 2007;67:3673–3682. [PubMed: 17440079]
12. Ma L, Lu MF, Schwartz RJ, Martin JF. Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning. Development (Cambridge, England) 2005;132:5601–5611.
13. Boyer AS, Ayerinskas II, Vincent EB, McKinney LA, Weeks DL, Runyan RB. TGFbeta2 and TGFbeta3 have separate and sequential activities during epithelial-mesenchymal cell transformation in the embryonic heart. Developmental biology 1999;208:530–545. [PubMed: 10191064]
14. Camenisch TD, Molin DG, Person A, Runyan RB, Gittenberger-de Groot AC, McDonald JA, Klewer SE. Temporal and distinct TGFbeta ligand requirements during mouse and avian endocardial cushion morphogenesis. Developmental biology 2002;248:170–181. [PubMed: 12142029]

15. Bartram U, Molin DG, Wisse LJ, Mohamad A, Sanford LP, Doetschman T, Speer CP, Poelmann RE, Gittenberger-de Groot AC. Double-outlet right ventricle and overriding tricuspid valve reflect disturbances of looping, myocardialization, endocardial cushion differentiation, and apoptosis in TGF-beta(2)-knockout mice. Circulation 2001;103:2745–2752. [PubMed: 11390347]
16. Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. Development 1997;124:2659–2670. [PubMed: 9217007]
17. Cuevas BD, Abell AN, Johnson GL. Role of mitogen-activated protein kinase kinase kinases in signal integration. Oncogene 2007;26:3159–3171. [PubMed: 17496913]
18. Corson LB, Yamanaka Y, Lai KM, Rossant J. Spatial and temporal patterns of ERK signaling during mouse embryogenesis. Development 2003;130:4527–4537. [PubMed: 12925581]
19. Camenisch TD, Spicer AP, Brehm-Gibson T, Biesterfeldt J, Augustine ML, Calabro A Jr, Kubalak S, Klewer SE, McDonald JA. Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. J Clin Invest 2000;106:349–360. [PubMed: 10930438]
20. Lakkis MM, Epstein JA. Neurofibromin modulation of ras activity is required for normal endocardial-mesenchymal transformation in the developing heart. Development 1998;125:4359–4367. [PubMed: 9778496]
21. Niihori T, Aoki Y, Narumi Y, Neri G, Cave H, Verloes A, Okamoto N, Hennekam RC, Gillessen-Kaesbach G, Wieczorek D, Kavamura MI, Kurosawa K, Ohashi H, Wilson L, Heron D, Bonneau D, Corona G, Kaname T, Naritomi K, Baumann C, Matsumoto N, Kato K, Kure S, Matsubara Y. Germline KRAS and BRAF mutations in cardio-facio-cutaneous syndrome. Nat Genet 2006;38:294–296. [PubMed: 16474404]
22. Stevens MV, Parker P, Vaillancourt RR, Camenisch TD. MEKK4 regulates developmental EMT in the embryonic heart. Dev Dyn 2006;235:2761–2770. [PubMed: 16894626]
23. Molin DG, Bartram U, Van der Heiden K, Van Iperen L, Speer CP, Hierck BP, Poelmann RE, Gittenberger-de-Groot AC. Expression patterns of Tgfbeta1-3 associate with myocardialisation of the outflow tract and the development of the epicardium and the fibrous heart skeleton. Dev Dyn 2003;227:431–444. [PubMed: 12815630]
24. Fritz A, Brayer KJ, McCormick N, Adams DG, Wadzinski BE, Vaillancourt RR. Phosphorylation of serine 526 is required for MEKK3 activity, and association with 14-3-3 blocks dephosphorylation. The Journal of biological chemistry 2006;281:6236–6245. [PubMed: 16407301]
25. Rodgers LS, Lalani S, Hardy KM, Xiang X, Broka D, Antin PB, Camenisch TD. Depolymerized hyaluronan induces vascular endothelial growth factor, a negative regulator of developmental epithelial-to-mesenchymal transformation. Circ Res 2006;99:583–589. [PubMed: 16931798]
26. Potts JD, Runyan RB. Epithelial-mesenchymal cell transformation in the embryonic heart can be mediated, in part, by transforming growth factor beta. Developmental biology 1989;134:392–401. [PubMed: 2744239]
27. Tamiolakis D, Papadopoulos N, Sivridis E, Anastasiadis P, Karamanidis D, Romanidis C, Kotini A, Bounovas A, Simopoulos C. Expression of the intermediate filament vimentin and fibrillar proteins of the extracellular matrix related to embryonal heart development. Clinical and experimental obstetrics & gynecology 2001;28:193–195.
28. Desgrosellier JS, Mundell NA, McDonnell MA, Moses HL, Barnett JV. Activin receptor-like kinase 2 and Smad6 regulate epithelial-mesenchymal transformation during cardiac valve formation. Developmental biology 2005;280:201–210. [PubMed: 15766759]
29. Abbasi S, Su B, Kellems RE, Yang J, Xia Y. The essential role of MEKK3 signaling in angiotensin II-induced calcineurin/nuclear factor of activated T-cells activation. The Journal of biological chemistry 2005;280:36737–36746. [PubMed: 16126726]
30. Brown CB, Boyer AS, Runyan RB, Barnett JV. Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart. Science 1999;283:2080–2082. [PubMed: 10092230]
31. Romano LA, Runyan RB. Slug is an essential target of TGFbeta2 signaling in the developing chicken heart. Developmental biology 2000;223:91–102. [PubMed: 10864463]

32. Kern CB, Hoffman S, Moreno R, Damon BJ, Norris RA, Krug EL, Markwald RR, Mjaatvedt CH. Immunolocalization of chick periostin protein in the developing heart. The anatomical record 2005;284:415–423. [PubMed: 15803479]
33. Norris RA, Moreno-Rodriguez RA, Sugi Y, Hoffman S, Amos J, Hart MM, Potts JD, Goodwin RL, Markwald RR. Periostin regulates atrioventricular valve maturation. Developmental biology 2008;316:200–213. [PubMed: 18313657]
34. Barnett JV, Moustakas A, Lin W, Wang XF, Lin HY, Galper JB, Maas RL. Cloning and developmental expression of the chick type II and type III TGF beta receptors. Dev Dyn 1994;199:12–27. [PubMed: 8167376]
35. Rivera-Feliciano J, Tabin CJ. Bmp2 instructs cardiac progenitors to form the heart-valve-inducing field. Developmental biology 2006;295:580–588. [PubMed: 16730346]
36. Norris RA, Kern CB, Wessels A, Moralez EI, Markwald RR, Mjaatvedt CH. Identification and detection of the periostin gene in cardiac development. The anatomical record 2004;281:1227–1233. [PubMed: 15532025]
37. Yang J, Boerm M, McCarty M, Bucana C, Fidler IJ, Zhuang Y, Su B. Mekk3 is essential for early embryonic cardiovascular development. Nat Genet 2000;24:309–313. [PubMed: 10700190]
38. Deng Y, Yang J, McCarty M, Su B. MEKK3 is required for endothelium function but is not essential for tumor growth and angiogenesis. Am J Physiol Cell Physiol 2007;293:C1404–C1411. [PubMed: 17687003]
39. Li H, Wicks WD. Retinoblastoma protein interacts with ATF2 and JNK/p38 in stimulating the transforming growth factor-beta2 promoter. Arch Biochem Biophys 2001;394:1–12. [PubMed: 11566021]
40. Gaussin V, Van de Putte T, Mishina Y, Hanks MC, Zwijsen A, Huylebroeck D, Behringer RR, Schneider MD. Endocardial cushion and myocardial defects after cardiac myocyte-specific conditional deletion of the bone morphogenetic protein receptor ALK3. Proc Natl Acad Sci U S A 2002;99:2878–2883. [PubMed: 11854453]
41. Inai K, Norris RA, Hoffman S, Markwald RR, Sugi Y. BMP-2 induces cell migration and periostin expression during atrioventricular valvulogenesis. Developmental biology 2008;315:383–396. [PubMed: 18261719]
42. Yamagishi T, Nakajima Y, Miyazono K, Nakamura H. Bone morphogenetic protein-2 acts synergistically with transforming growth factor-beta3 during endothelial-mesenchymal transformation in the developing chick heart. J Cell Physiol 1999;180:35–45. [PubMed: 10362015]
43. Letterio JJ, Geiser AG, Kulkarni AB, Roche NS, Sporn MB, Roberts AB. Maternal rescue of transforming growth factor-beta 1 null mice. Science 1994;264:1936–1938. [PubMed: 8009224]
44. Butcher JT, Norris RA, Hoffman S, Mjaatvedt CH, Markwald RR. Periostin promotes atrioventricular mesenchyme matrix invasion and remodeling mediated by integrin signaling through Rho/PI 3-kinase. Developmental biology 2007;302:256–266. [PubMed: 17070513]

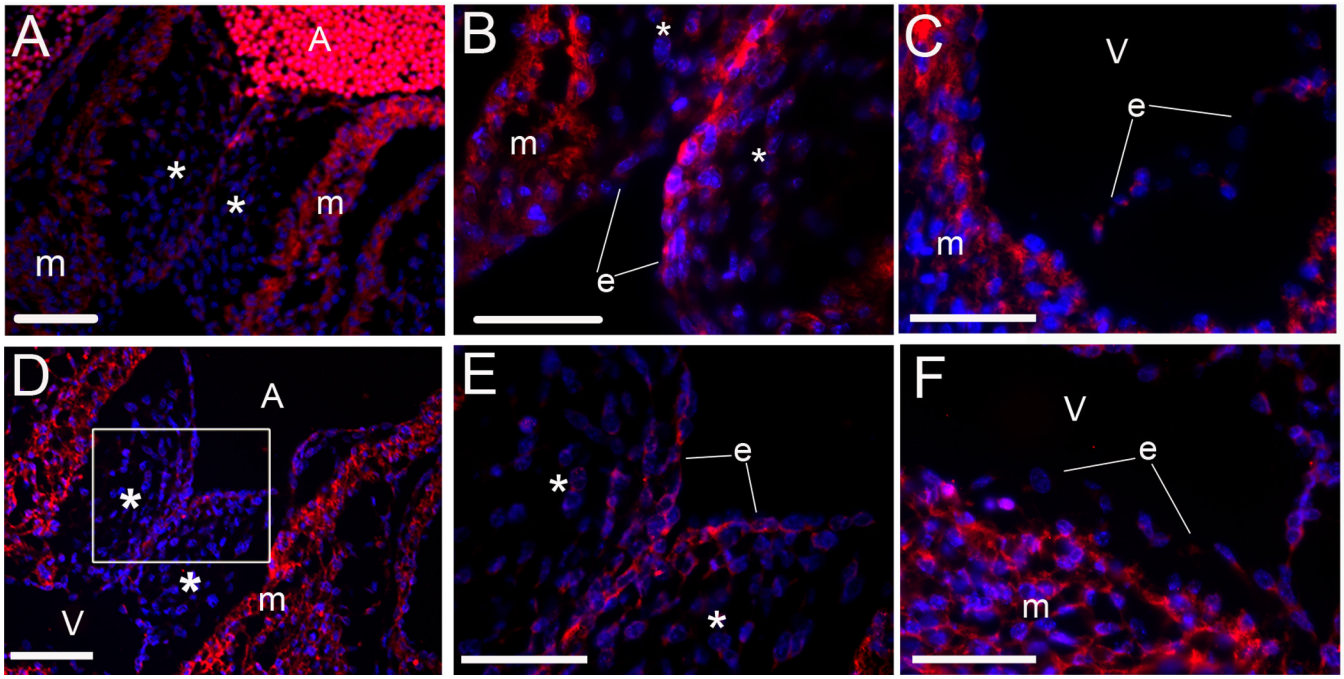


Figure 1.

MEKK3 detected in embryonic heart during endocardial cushion EMT. Immunofluorescent-detection of MEKK3 to E9.5 (A, B, C) and E10.5 hearts (D, E, F). MEKK3 protein at E9.5 is located to the AVC cushion endocardium (B), but there is minute to no detection to the ventricular endocardium (C). MEKK3 – red, Hoescht dye (nuclei) – blue. A = atrium, V = ventricle, *, asterisks = AVC endocardial cushion, m = myocardium, e = endocardium. Scale bar in A and D = 100 microns; scale bar in B, C, E, F = 50 microns.

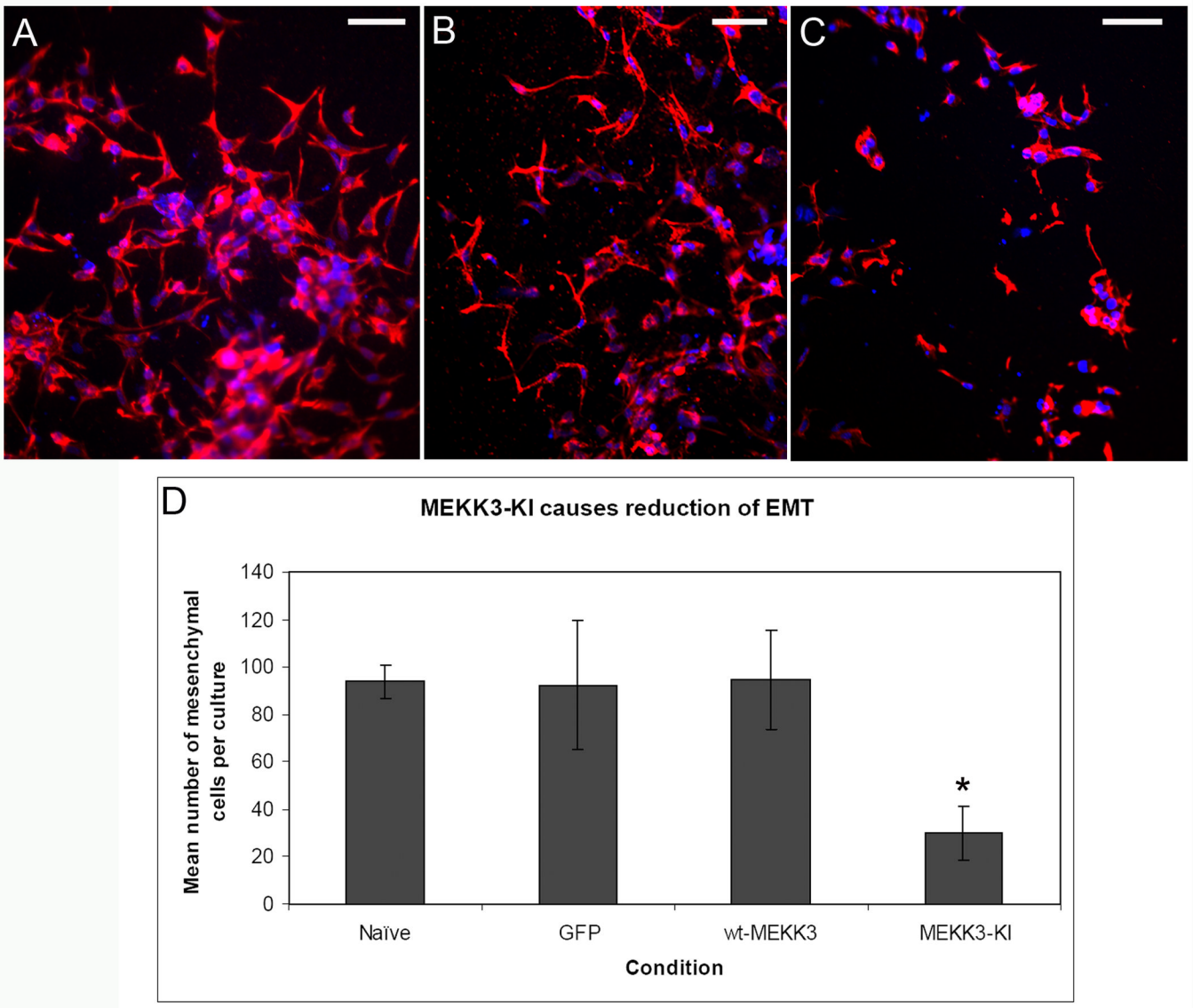


Figure 2.

Kinase inactive MEKK3 (MEKK3^{KI}) blocks EMT. AVC explants from E9.5 embryos under naïve conditions, or with Ad-GFP (A), wt-MEKK3 (B), or MEKK3^{KI} (C). Red – Vimentin, Blue – nuclear stain. Graph of vimentin-positive mesenchyme from these experiments (D). Naïve n = 5; GFP n = 11; wt-MEKK3 n = 9; MEKK3^{KI} n = 14. MEKK3^{KI} vs. GFP, *, $p < 0.0001$. Scale bar in A, B, C = 50 microns.

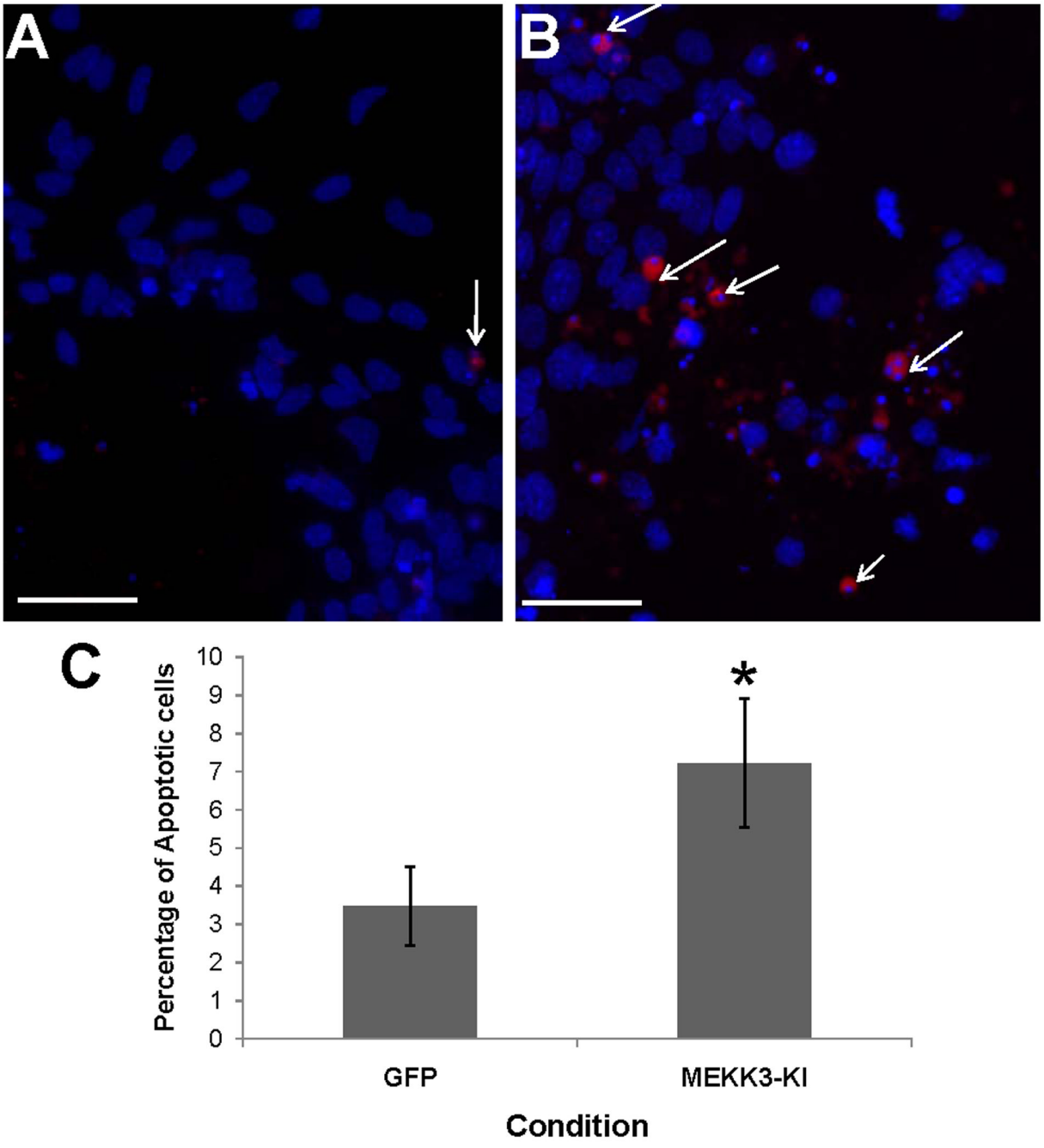


Figure 3.

MEKK3 activity is anti-apoptotic. Increased detection of active caspase-3, an indicator of apoptosis, in MEKK3^{KI} AVC explants compared to controls (Compare A and B). AVC explants from E9.5 embryos with GFP (A) or MEKK3^{KI} (B). Red – Active caspase-3, Blue – nuclear stain. Graph comparing active caspase-3 positive cells to total cells (C). GFP n = 7; MEKK3^{KI} n = 10. MEKK3^{KI} vs. GFP, *, p = 0.0077. Scale bar A, B = 50 microns.

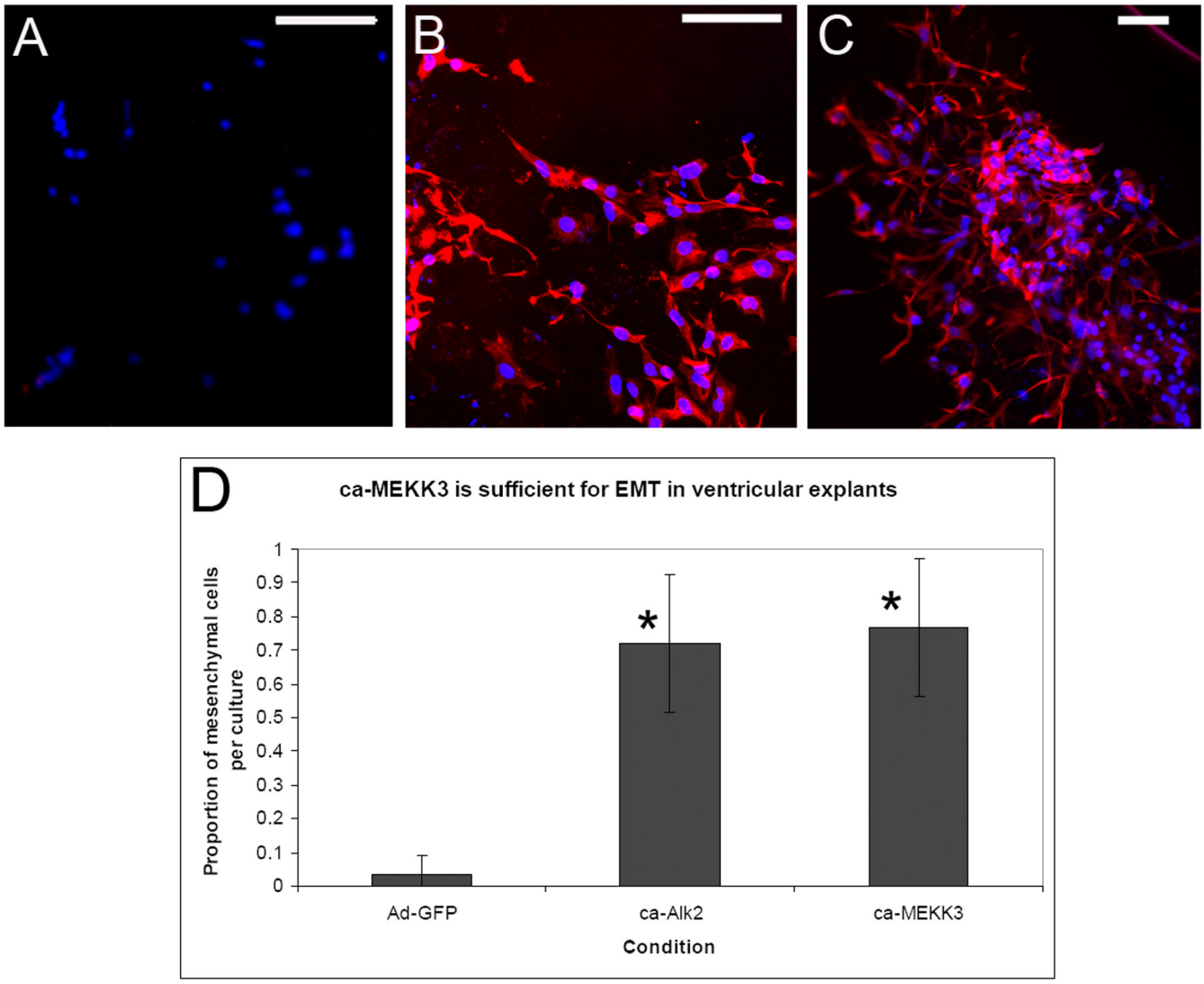


Figure 4.

ca-MEKK3 is sufficient for cardiac EMT. E9.5 endocardial explants from ventricles treated with Ad-GFP (A), ca-Alk2 (B), or ca-MEKK3 (C). Red – Vimentin, Blue – nuclear stain. Enumeration of vimentin-positive cells versus total cells in culture (D). GFP n = 12; ca-Alk2 n = 11; ca-MEKK3 n = 12. ca-Alk2 vs. GFP, $p < 0.01$; ca-MEKK3 vs. GFP, $p < 0.01$. Scale bar in A, B, C = 100 microns.

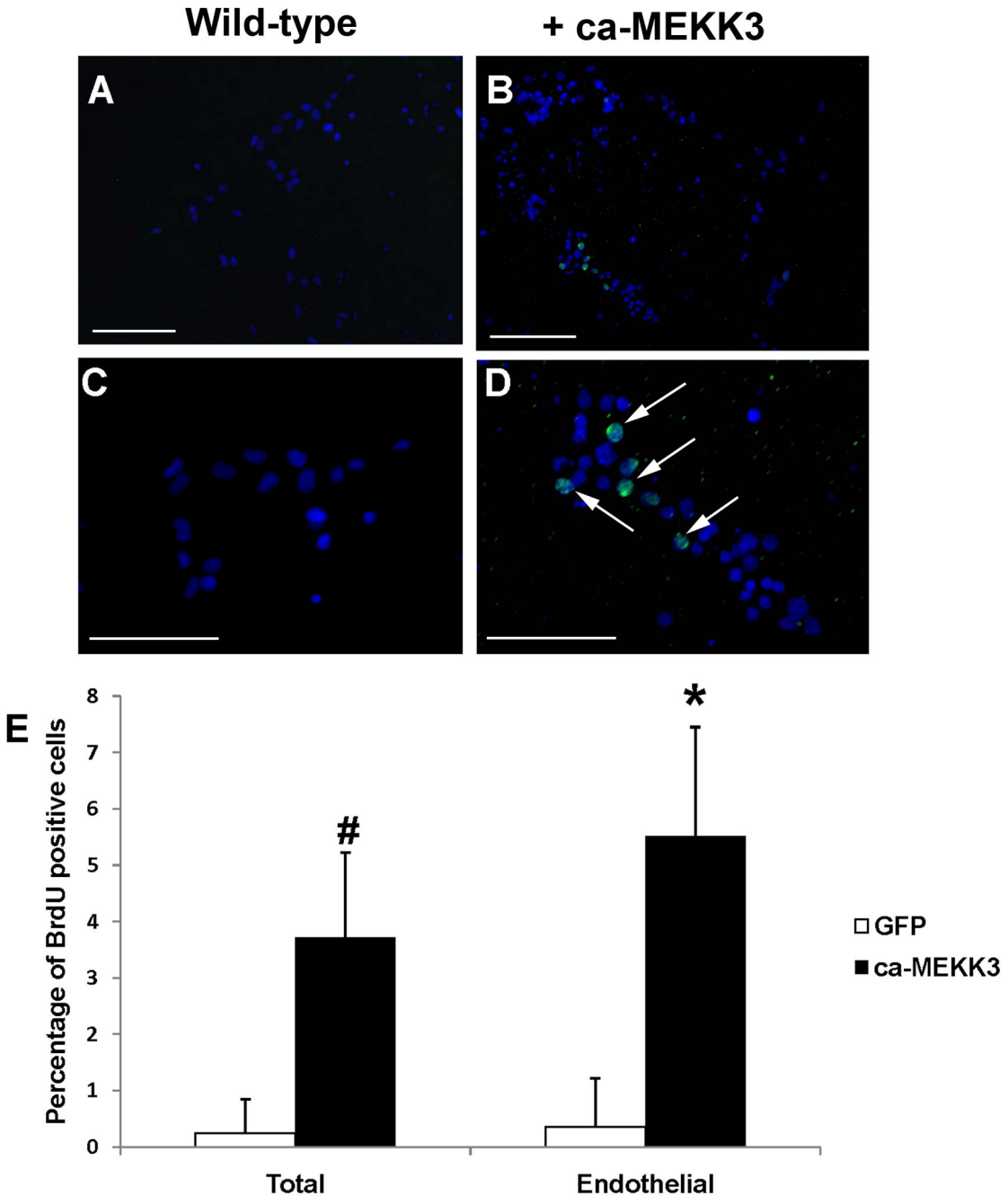


Figure 5.

MEKK3 increases cell proliferation. Elevated detection of BrdU-positive cells in E9.5 ca-MEKK3 ventricular explants (B) compared to controls (A). Enumeration of BrdU-positive cells vs. total cells and BrdU-positive endothelial cells vs. total endothelial cells (E). Naïve n = 6; ca-MEKK3 n = 5. #, p = 0.0037; *, p = 0.0077. Scale bar A, B = 100 microns; C, D = 50 microns.

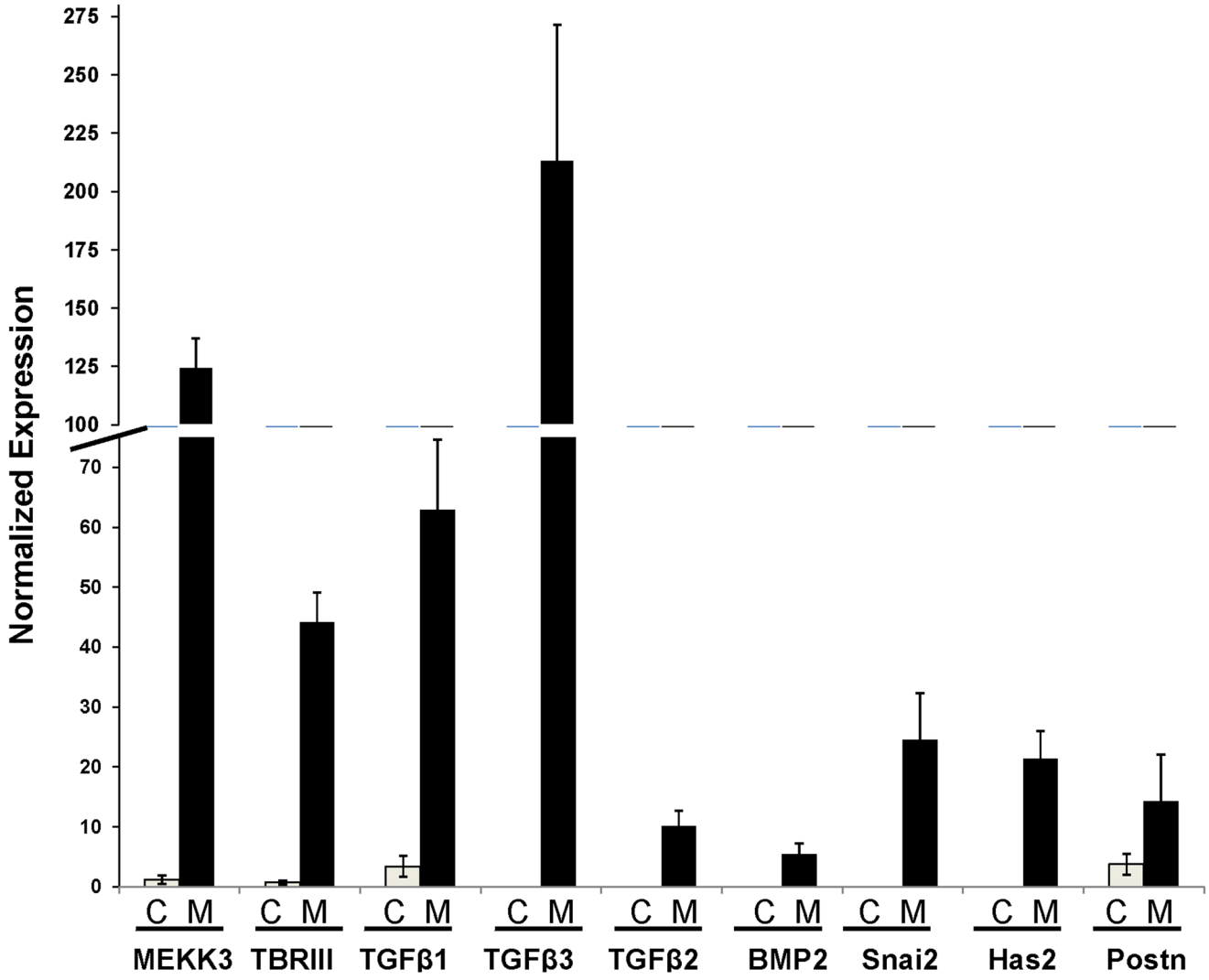
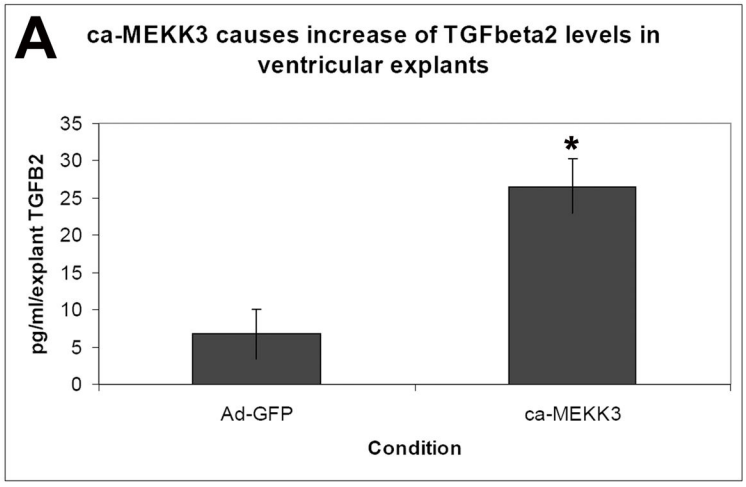


Figure 6.

ca-MEKK3 induces endocardial EMT. Detection of EMT-related genes is relative to the housekeeping gene, Aminolevulinate, delta-, synthase 1 (Alas1). Genes examined were MEKK3, TGFβ type III receptor (TβRIII), TGFβ1, TGFβ3, TGFβ2, BMP2, Snail2, Has2, and Periostin. Ad-GFP control (C) cultures (clear bars) versus ca-MEKK3 (M) cultures (filled bars). Primers to the MEKK3 kinase domain were used to check transfection of ca-MEKK3. Normalized expression was calculated using mean control value for gene analyzed.



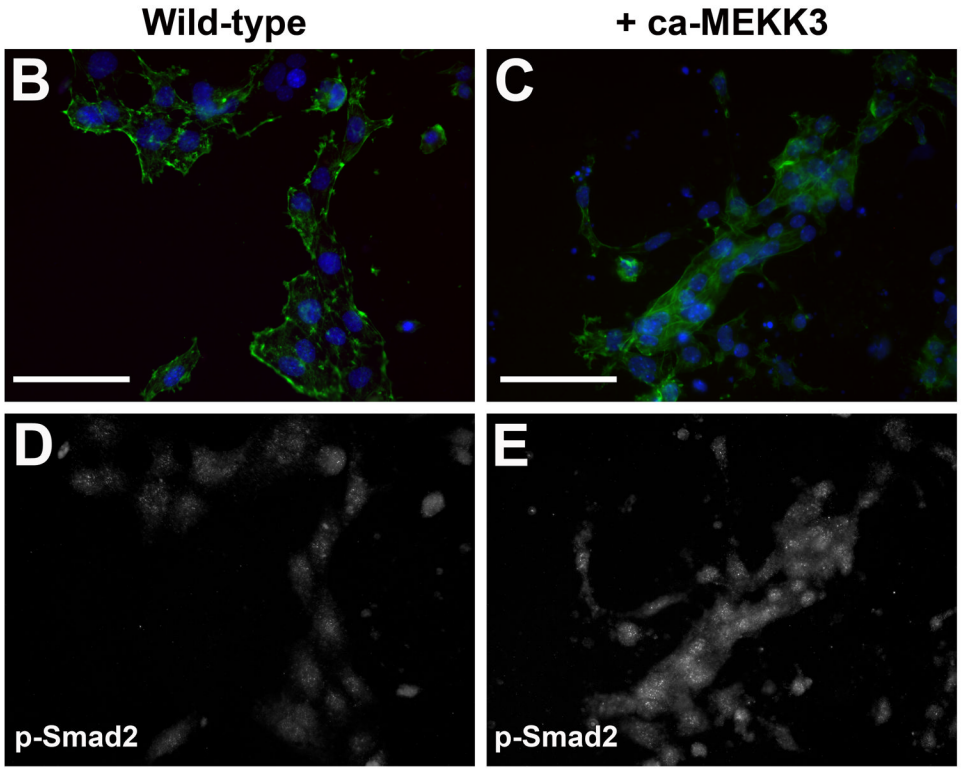


Figure 7.

ca-MEKK3 induces production of TGFβ2 in ventricular endocardium explants. Detection of TGFβ2 by ELISA in Ad-GFP conditions (clear column, n =24) and ca-MEKK3 (filled column, n = 23). Graph of ELISA data (A) (*, $p = 1.02*10^{-5}$; 99% CI). p-Smad2 levels increase with ca-MEKK3 (Compare D and E). Ventricular explants cultures with GFP only (B, D) or ca-MEKK3 (C, E). Green – Filamentous actin, Blue – nuclear stain. Immunofluorescent detection of p-Smad2 shown in monochromatic image (D and E). For p-Smad2 experiments, GFP n = 3; ca-MEKK3 n = 3. Scale bar in B, C, D, E = 50 microns.

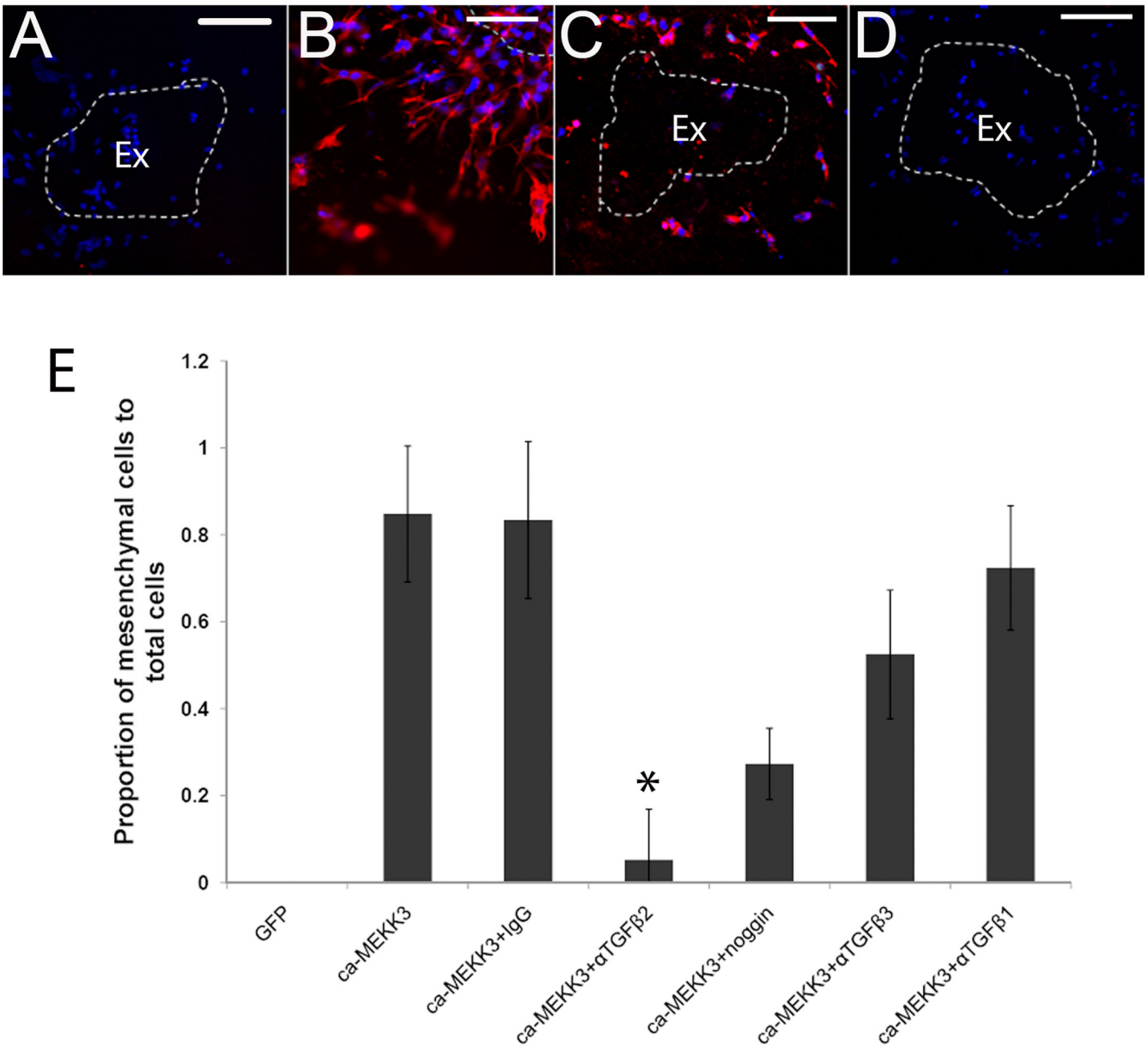


Figure 8.

MEKK3 drives EMT in a TGFβ2-dependent manner. Neutralizing TGFβ2 with anti-TGFβ2 blocks ca-MEKK3 induced EMT in ventricular explants. Images of Naïve (A), ca-MEKK3 (B), ca-MEKK3 with control isotype-matched IgG (C), and ca-MEKK3 with anti-TGFβ2 blocking antibody (D). Graph of TGFβ2 blocking experiments (E) (ca-MEKK3 vs. ca-MEKK3 + TGFβ2 neutralizing antibody, *, $p = 0.006$; 99% CI; Compare to ca-MEKK3 + anti-TGFβ2; #, $p < 0.01$; Compare to ca-MEKK3, $, $p > 0.05$). Naïve n = 5; ca-MEKK3 n = 3; ca-MEKK3 + control IgG n = 4; ca-MEKK3 + TGFβ2 antibody n = 11; ca-MEKK3 + noggin n = 11; ca-MEKK3 + anti-TGFβ3 n = 10; ca-MEKK3 + anti-TGFβ1, n = 9. Vimentin – Red, Nuclear stain – Blue. Ex = explant, dashed line represents outline of initial explant. Scale bar = 100 microns.