MECHANISMS OF HAS2 REGULATION AND HYALURONAN SIGNALING DURING EMBRYONIC DEVELOPMENT

By

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LIST OF ABBREVIATIONS

ECM = Extracellular Matrix

EGF = Epidermal Growth Factor

EMT = Epithelial to Mesenchymal Transition

ERK = Extracellular Signal Regulated Kinase

FACE = Fast Activated Cell Based Enzyme-Linked Immunosorbent Assay

HA = Hyaluronan

Has2 = Hyaluronan Synthase 2

HMW-HA = High Molecular Weight Hyaluronan

IF = Immunofluorescence

IP = Immunoprecipitation

KI = Kinase Inactive

LMW-HA = Low Molecular Weight Hyaluronan

MAPK = Mitogen Activated Protein Kinase

MAP3K = Mitogen Activated Protein Kinase Kinase Kinase

NFκB = Nuclear Factor Kappa B

RT-PCR = Real Time Polymerase Chain Reaction

SEAP = Secreted Alkaline Phosphatase

SRE = Serum Response Element

TGFβ = Transforming Growth Factor Beta

WB = Western Blot

ABSTRACT

The cardiovasculature is the first functional system in the developing embryo and as such, it plays a crucial role in the proper nourishment and formation of all other body regions and organs. Malformation or malfunction of this system can lead to a series of conditions collectively known as cardiovascular diseases, which are a major cause of death around the world, claiming 17.5 million human lives a year. A detailed understanding the mechanisms that regulate cardiac morphogenesis is necessary to provide us with clues of what goes awry in disease states and to develop possible strategies for diagnostics and treatment of these problems.

There is a wide variety of processes and molecules that have been identified to date as important players for the proper formation of the cardiovasculature. One of these molecules is Hyaluronan synthase 2 (Has2), a membrane protein in charge of assembling the glycosaminoglycan hyaluronan (HA). Mouse embryos lacking Has2 do not produce HA and display abnormalities such as absence of endocardial cushions, a disarrayed vascular network, and growth retardation, leading to death by embryonic day E 9.5. Thus, Has2 and HA are necessary for the early stages of heart formation, but many questions remain to be answered in regards to their mechanism of action and their role in later events such as the formation of the coronary vessels. Our current study addresses these questions in vitro employing two cell lines. NIH-3T3 cells are used as a model of mesenchymal endocardial cushion cells while

epicardial cells are used to assess Has2 and HA function in embryonic cells with an epithelial phenotype.

Here we show that HA induces biological activity in embryonic cells in a manner that is dependent on its molecular size, with high molecular weight HA (HMW-HA), but not low molecular weight HA (LMW-HA), being able to affect cellular behavior. HMW-HA induces invasion of NIH-3T3 cells while it promotes differentiation and invasion of epicardial cells.

We also demonstrate that stimulation of cells with HMW-HA promotes the association of MEKK1 with the HA receptor CD44 and induces MEKK1 phosphorylation. This leads to the activation of two distinct pathways, one ERK-dependent and another NFκB-dependent. Although both cells lines show activation of these cascades, the ERK-dependent pathway is more prominent in epicardial cells while the NFκB-dependent pathway is favored in NIH-3T3 cells. Blockade of CD44, transfection with a kinase inactive MEKK1 construct or the use of ERK1/2 and NFκB inhibitors significantly abrogates the cellular response to HMW-HA. Together, these findings suggest an important role for HA in the regulation of embryonic cell fate via activation of MEKK1 signaling mechanisms.

Finally, we have elucidated a novel functional connection between growth factor signaling, endogenous HA production and the regulation of cellular responses. Specifically, we show that both TGFβ2 and EGF induce Has2 expression and/or phosphorylation, although distinct intracellular signals are activated for each growth

factor. While TGFβ2 governs Has2 via MEKK3-dependent mechanisms, EGF does not require MEKK3 and does not induce Has2 expression as robustly as TGFβ2. Increased Has2 activity as a result of TGFβ2 and EGF stimulation leads to enhanced HA synthesis. These increased endogenous levels of HA are coincident with enhanced cellular differentiation and invasion. Taken together, these findings underscore how EGF, TGFβ2 and HA signals are integrated to form highly complex networks that are crucial for the proper formation of organs and tissues during development.

CHAPTER 1

BACKGROUND, PURPOSE AND AIMS

1.1. The EMT Process and Cardiovascular Development

The vertebrate heart is a complex organ and its proper formation and function requires a multitude of specific cell interactions. Elucidation of the signaling mechanisms that drive the differentiation, migration, and assembly of these cells into different components such as muscle tissue, the conduction system and the coronary vasculature is important to further understand the etiology of congenital heart defects. These ailments constitute a significant public health problem as they affect approximately 50 out of every 1,000 babies born in the United States (110). This knowledge of developmental cellular mechanisms will also provide insight into cardiovascular regeneration processes which has implications for the treatment of adult ailments such as coronary artery disease and mitral valve disease.

1.1.1. Major morphological events in heart formation:

In the mammalian embryo, heart formation begins when a population of cells from the anterior lateral plate mesoderm becomes committed to a cardiogenic fate in response to inductive signals from the adjacent endoderm. These group of cells, collectively known as the cardiac crescent, migrate toward the middle ventral part of the embryo to form the linear heart tube (Fig. 1.1 A,B) (147). This initial primitive tube is composed of an inner layer known as the endocardium and an outside layer,

termed the myocardium. The next step involves rearrangement of the heart into a two-chambered tube with a left-right orientation (Fig. 1.1C). This is achieved with a rightward looping that realigns the future ventricles so that the part destined to become the right ventricle lies anterior to the portion that will become the left ventricle (71). Meanwhile, a subset of cells from the endocardium gives rise to the endocardial cushions. This region undergoes a process known as epithelial to mesenchymal transition (EMT) to form the heart valves and septae, which divide the organ into four chambers (Fig 1.1D) (162). These steps are summarized in figure 1.1.

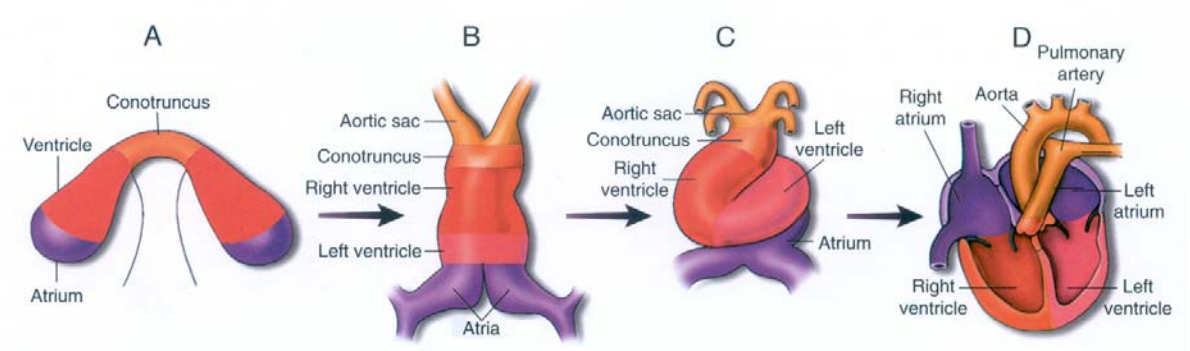

Figure 1.1. Schematic diagram of cardiac morphogenesis in mammals. (A) Formation of two symmetrical regions of cardiogenic mesoderm. (B) Fusion of cardiogenic areas into a single-chambered tube. (C) Rightward cardiac looping. (D) Septation and formation of the four-chambered heart. Adapted from Harvey et al (51).

Shortly after heart looping and as the organ continues to develop, another important event takes place: the formation of the coronary vessels. Here, a group of mesothelial cells, derived from the proepicardial organ, migrates from the ventral side of the embryo and adheres to the surface of the heart. The resulting outer layer that covers the myocardium is termed the epicardium (56). A subset of epicardial cells

then undergoes EMT and migrates into the myocardium to give rise to the coronary vasculature (Fig 1.2). This intricate system of vessels is crucial to provide nourishment and signals that are necessary for proper maturation and functioning of the heart muscle throughout an individual's life. In addition to their involvement in coronary vascular formation, epicardium-derived cells can also give rise to cardiac fibroblasts and smooth muscle cells that contribute to the formation of the atrioventricular valves and the fibrous heart skeleton (83). Furthermore, recent studies have identified myocardial cell lineages in the ventricular septum and wall that are derived from epicardial progenitors (17). Thus, the epicardium provides an important population of pluripotent cells that participate in the formation of multiple tissues within the cardiovascular system.

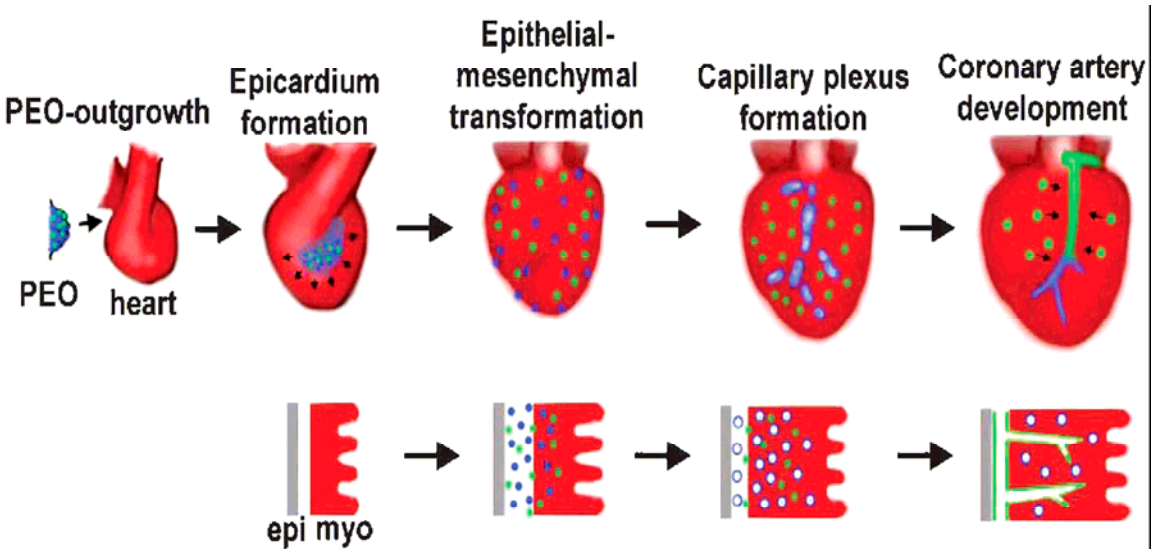

Figure 1.2. Development of the coronary vessels. Cells from the proepicardial organ (PEO) migrate to the heart and surround it, giving rise to the epicardium. A subset of epicardial cells then undergo EMT and invade into the myocardium to form the coronary vasculature. Adapted from Reese et al (119).

1.1.2. The importance of EMT and the mechanisms through which it occurs:

EMT is vital to create new cell layers and tissues throughout multiple stages of cardiovascular development, including valvuloseptal and coronary vascular morphogenesis. However, the EMT process participates in many other events during embryogenesis such as implantation of the embryo into the uterus, generation of the three germ layers during gastrulation and formation of the neural crest, which gives rise to several components of the nervous system (1, 38, 111). Furthermore, EMT is critical for normal physiological events that occur post-development such as wound healing and tissue regeneration and it also contributes to the progression of disease states such as organ fibrosis and cancer (70).

In the context of embryogenesis, EMTs are driven by the evolutionary need to remodel and diversify tissue originally derived from a single cell so that specific organized cells can perform specialized functions. The EMT process allows epithelial cells to undergo multiple biochemical changes to acquire a mesenchymal phenotype. Mesenchymal cells differ from their epithelial counterparts in that they lack intercellular junctions, possess enhanced invasive capacity, have elevated resistance to apoptosis and increased production of extracellular matrix (ECM) components and proteases (69, 149).

EMT involves several steps such as detachment of epithelial cells from the basement membrane, proliferation, differentiation into a mesenchymal phenotype and invasion through the ECM (Fig. 1.3). The initiation and completion of each of these

steps requires the activation and integration of complex intracellular pathways in response to specific extracellular stimuli. Among these molecules, those that are considered relevant for developmental EMT include growth factors such as EGF and TGFβ2 and the extracellular matrix component hyaluronan (18, 30, 129). Thus, our primary focus is understanding how these signals are interconnected and regulated in epithelial as well as in mesenchymal cells.

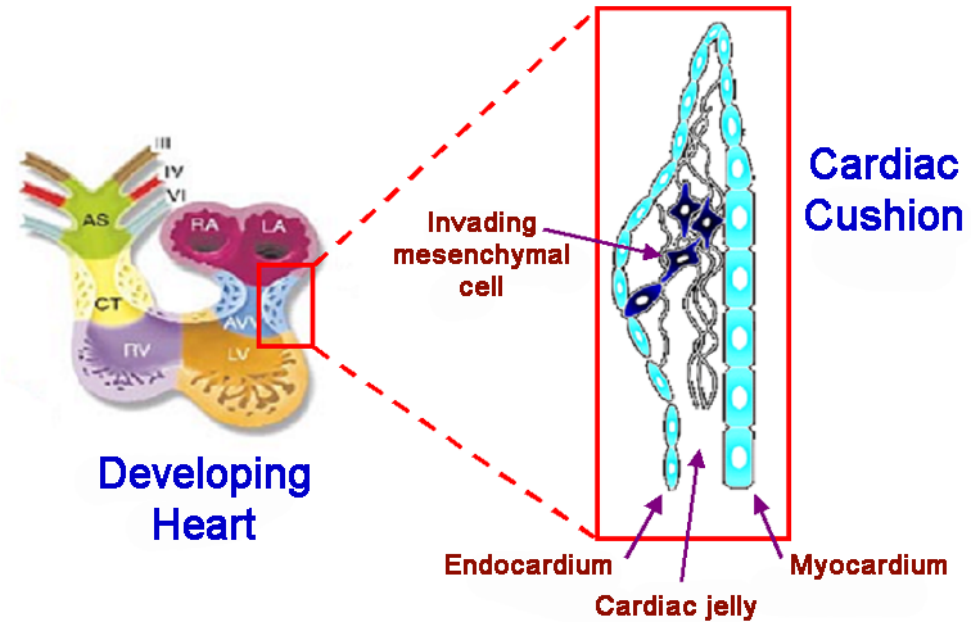

Figure 1.3. The EMT process in cardiac cushions. Epithelial cells from the innermost layer of the heart or endocardium detach, proliferate and differentiate into mesenchymal cells. Then, they invade through the space known as the cardiac jelly towards the myocardium, eventually giving rise to the heart valves and septa.

1.2. Growth Factors and their Contribution to Cardiovascular Formation

During cardiovascular development, the three layers of the heart (endocardium, myocardium and epicardium) secrete multiple growth factors that exert regulatory effects in an autocrine and paracrine manner (132, 138). Among these molecules, transforming growth factor beta 2 (TGFβ2) and epidermal growth factor (EGF) are of particular interest as multiple in vitro and in vivo studies have shown that these proteins and their intracellular effectors are able to regulate multiple steps of the EMT process in the heart (96, 100). Furthermore, the importance of TGFβ2, EGF, and the signaling pathways that they govern is underscored by the linkage of mutations in components of such pathways with human congenital heart defects.

1.2.1. EGF and its downstream signals:

EGF is the prototypic member of the EGF family, which includes six structurally related polypeptides: EGF, TGF-α, amphiregulin (AR), heparin-binding EGF (HB-EGF), betacellulin (BTC), and epiregulin (EPR). This group of proteins shares two main characteristics: the first is that they all contain a three-loop compact structure, termed the EGF-like domain, that is formed by disulfide bridges between 6 conserved cysteines and provides a specific conformation that is important for ligand binding to cell surface molecules. The second common characteristic is that all EGF family members are first synthesized as transmembrane proteins and their soluble forms are proteolytically derived through a process known as ectodomain shedding (79). The proteolytic cleavage of EGF from the membrane is performed by ADAM (a desintegrin and metalloprotease) proteins and is required for EGF activation (127).

EGF initiates cellular responses by binding to the EGF receptor (EGFR/HER1/ErbB1), which then forms homodimers or heterodimers with related ErbB family members such as ErbB2/HER2. Receptor dimerization induces activation of the EGFR tyrosine kinase domain and phosphorylation on multiple sites of the receptor cytoplasmic tails. This results in the assembly of signaling complexes and stimulation of numerous downstream pathways, such as the Ras/Raf, PI3K, PLC and STAT-dependent cascades (Fig. 1.4) (133).

The Ras/Raf pathway is activated in response to EGF by recruitment of the adaptor proteins GRB2 or SHP2 and the guanine exchange factor SOS to the phosphorylated receptors. SOS then causes the small G protein Ras to release GDP and exchange it for GTP, which results in Ras activation. Next, Ras binds to Raf and promotes sequential activation of the kinases Raf, MEK and ERK1/2. This leads to the induction of responses such as survival, proliferation, apoptosis and migration, depending on the cellular context (117).

EGF induces the PI3K/Akt signaling pathway through recruitment of PI3K to the plasma membrane. Activated PI3K then catalizes the transfer of a phosphate group from ATP to phosphatidylinositol generating phosphatidylinositol phosphate (PIP). PIP then binds proteins with a pleckstrin homology (PH) domain, such as Akt, and facilitates their phosphorylation in the plasma membrane. Activation of Akt leads to inactivation of pro-apoptotic molecules and induction of anti-apoptotic genes, and thus it is important for cell survival (139).

EGF-dependent activation of PLC is initiated by binding of PLC to EGF receptors and subsequent phosphorylation. Once active, PLC cleaves membrane bound phosphotidylinositol bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). These products then collaborate to activate PKC, with IP3 inducing its translocation to the cell membrane and DAG promoting PKC phosphorylation. This pathway is important for cell survival but has also been shown to increase the sensitivity of some cell types to radiation therapy (98).

STAT proteins are activated upon EGF stimulation either through direct phosphorylation by EGF receptors or through activation of the intracellular kinase Src which then phosphorylates STAT (78). Activation of this pathway results in proliferation, cell cycle progression and inhibition of apoptosis (116).

Induction of either the Ras/Raf, PI3K, PLC or STAT pathway by EGF is not an isolated event and in most cases a complex intracellular network is activated to relay EGF signals. For example, in breast cancer cells, EGF can simultaneusly activate the Ras/Raf and PI3K cascades and crosstalk between these molecules facilitates cell survival and increased proliferation (65). Thus, it is likely that many other signaling effectors may participate in EGF-mediated cellular responses but are yet to be identified.

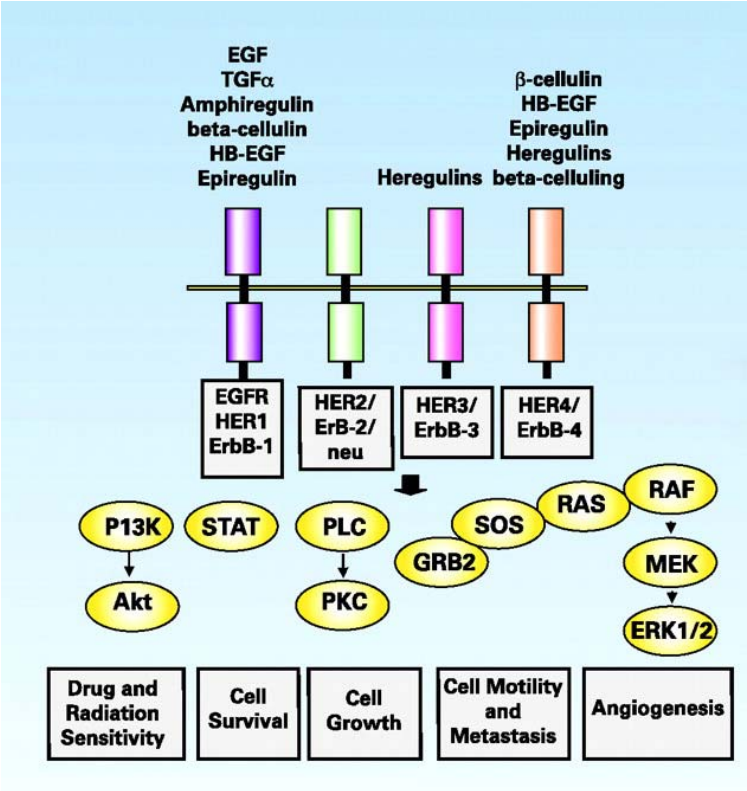

Figure 1.4. EGF family ligands and their downstream signaling effectors. EGF ligands interact with ErbB receptors to induce intracellular signals and promote a variety of biological responses. Adapted from Ono et al (104).

Although EGF knockout mice do not exhibit any overt abnormalities, EGF has been shown to be important for cellular proliferation, differentiation and invasion, suggesting that this molecule participates in the regulation of EMT in collaboration with other signals (133, 159). Also, mouse embryos lacking EGFR die around birth and have enlarged cardiac valves while mice with a disruption of the ErbB2 gene die by E10.5 due to malformation of the cardiac trabeculae (62, 81). These gene targeting studies indicate a role for EGFR and ErbB2 in cardiovascular formation and maturation.

Several intracellular molecules that regulate or relay signals initiated by EGF and its receptors have also been identified as important for cardiovascular development. Mutations of the tyrosine phosphatase SHP2, which mediates ErbB receptor-induced activation of Ras and PI3K, have been found to cause Noonan syndrome (148). This ailment is the most common single-gene cause of congenital heart disease in humans and is characterized by short stature, facial dismorphia and cardiac abnormalities such as hypertrophic cardiomyopathy and atrioventricular septal defects (3). Mutations in the K-Ras and SOS gene have also been linked to Noonan syndrome while mutations in H-Ras, B-Raf and MEK1/2 have been associated with the less frequent but related Costello syndrome, which also exhibits cardiovascular malformations (4, 37, 135). These observations indicate that EGF-activated signaling pathways play a relevant role in the formation of the cardiovascular system.

1.2.2. TGFβ2 signal transduction and functions:

TGFβs are multifunctional cytokines that regulate diverse processes during development such as cell proliferation, differentiation, migration and survival (100). In mammals, the immediate TGFβ family is composed of three isoforms (TGFβ1, TGFβ2 and TGFβ3) which share extensive amino acid sequence homology (60-80%) and are highly conserved between species. All three isoforms are synthesized and secreted by cells as latent complexes that require proteolytic cleavage for their activation and binding to cell surface receptors (16). TGFβ proteins initiate their signals by binding to TGFβ receptors (TβRII and TβRI) which are then trans-phosphorylated to induce diverse intracellular cascades, including Smads and MAPKs (Fig. 1.5).

In the canonical Smad pathway, stimulation with TGFβ proteins leads to binding of Smad 2/3 to the TGFβ receptors, phosphorylation and oligomerization of Smad2/3 with the common mediatior Smad4. This complex then translocates into the nucleus where it directs the transcription of genes that regulate cell growth, differentiation, matrix production and apoptosis (53). In certain cell types, TGFβs also activate MAPKs such as p38, JNK and ERK independently of Smads to regulate genes important for cell proliferation and differentiation (41).

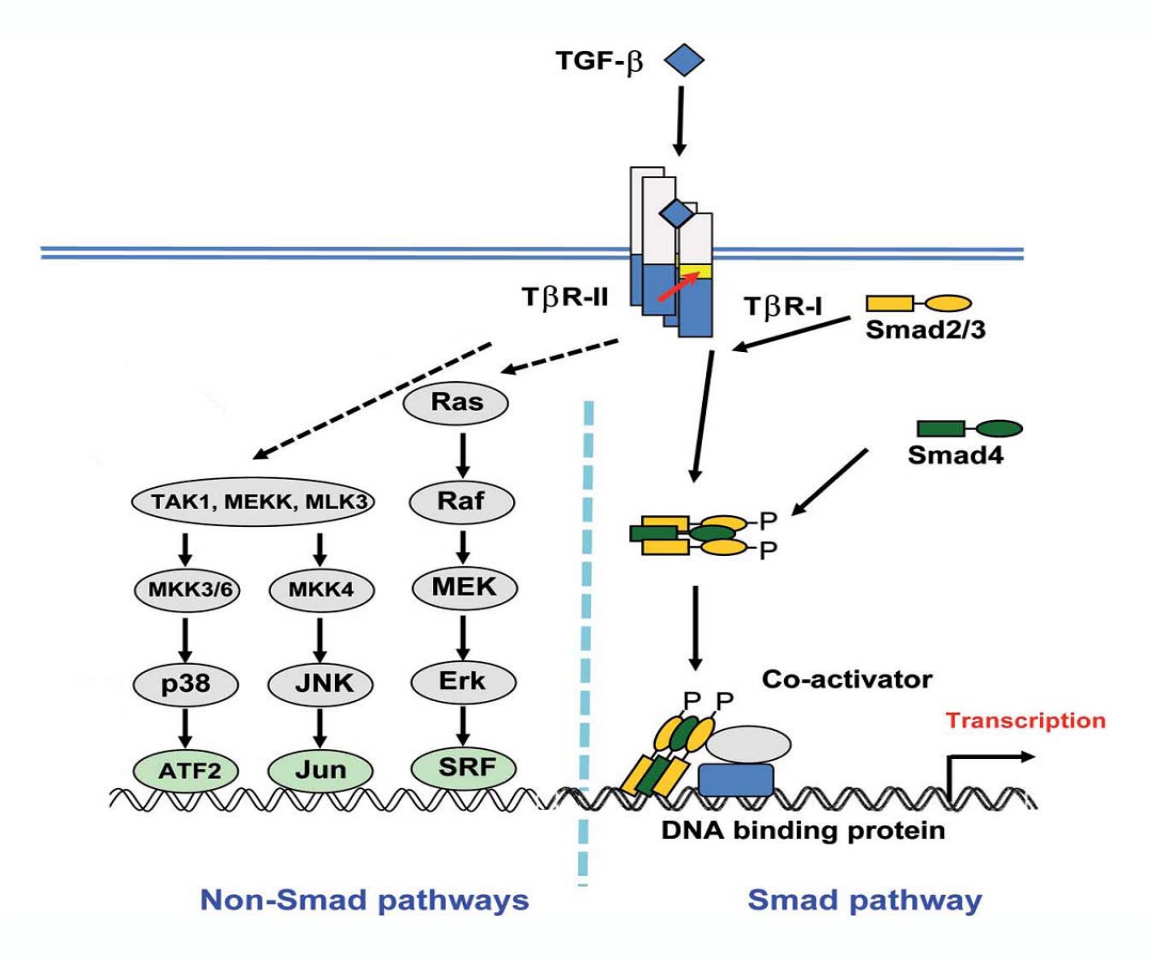

Figure 1.5. TGFβ-mediated signaling mechanisms. TGFβ interacts with TGFβ receptors in the cell surface and activates intracellular non-Smad and Smad-dependent pathways. Adapted from Miyazono et al (92).

In the developing heart, TGFβ2 is highly expressed in areas undergoing EMT such as the endocardial cushions and epicardium (94). Furthermore, mouse knockout studies have shown that TGFβ2, but not TGFβ1 or TGFβ3, is necessary for cardiovascular development. While TGFβ1 and TGFβ3 knockout mice do not show any cardiac defects, mice lacking TGFβ2 exhibit abnormal valve thickening, incomplete fusion of endocardial tissues and ventricular septal defects (67, 77, 129). Thus, the contribution of TGFβ2 to cardiac EMT is crucial for the proper formation and function of the heart.

Interestingly, several single point mutations of the type I and type II TGFβ receptors in humans have been associated congenital disorders such as Marfan syndrome II and Loeys-Dietz syndrome, which are characterized by severe cardiovascular defects (85, 93). This underlines an important role for the TGFβ pathways in cardiovascular morphogenesis. Furthermore, Marfan syndrome I is caused by mutations in fibrillin 1, a major constitutive element of conective tissue microfibrils which also interacts with extracellular matrix components such as hyaluronan (52, 102). This suggests possible interactions between TGFβ-mediated intracellular signals and extracellular molecules during the development of the cardiovasculature.

1.3. Hyaluronan Synthase 2 (Has2) and its Product Hyaluronan: Structural Properties and their Role in Heart Morphogenesis

Hyaluronan is an acidic, negatively charged, linear glycosaminoglycan, comprised of repetitive disaccharide units of *N*-acetyl-d-glucosamine (GlcNac) and d-glucuronic acid (GlcA) (Fig. 1.4). HA is readily found in most vertebrate organs and regions, although it is most abundant around soft connective tissues (40). For many years HA was thought of as a passive, space-filling component of the ECM, but in recent decades it has been established as an important molecule that contributes both to the structural and physiological characteristics of tissues.

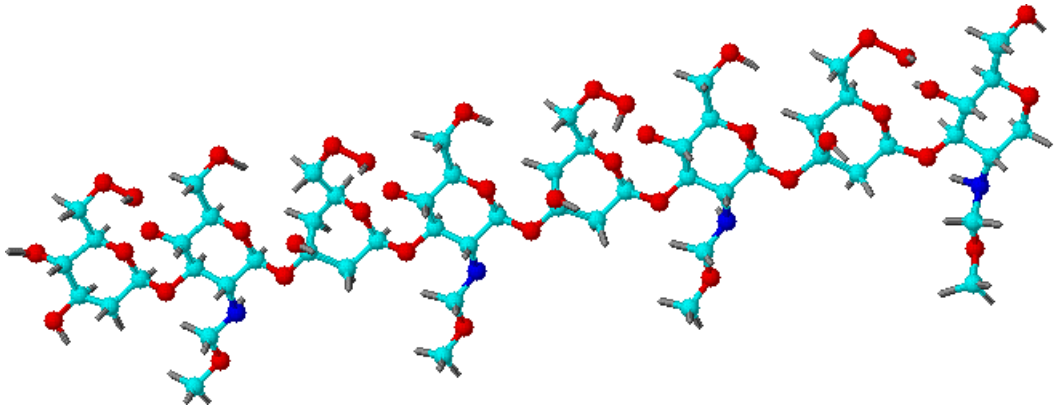

Figure 1.6. Structure of Hyaluronan. Hyaluronan is a linear carbohydrate composed of repeating glucuronic acid and N-acetylglucosamine disaccharides. Cyan= carbon, black = hydrogen, red= oxygen and blue= nitrogen. Image generated using the ChemSketch software (ACD Inc, Toronto, Canada).

Despite its relatively simple chemical composition, HA performs three distinct molecular functions. First, HA may interact in an autocrine manner with the surface of the same cell that produced it to form a hydrated pericellular matrix. Several cell types, including chondrocytes and oocytes, exhibit these HA-dependent matrices or “coats”, which provide a hydrated and protected environment that facilitates mitosis

and allows for the presentation of stimulating signals without the interference of the highly structured fibrous matrix (121, 156).

A second function is that HA interacts in a paracrine manner with other ECM molecules and neighboring cells to provide structural support. ECM proteins that are able to bind HA are collectively known as extracellular hyaladherins and include versican, aggrecan, neurocan, link protein, fibrinogen and trypsin inhibitor, which can interact with a single HA polymer to form large matrix complexes (47). These interactions are essential for the definition of size, shape and form of many tissues, such as cartilage, skin and smooth muscle (142).

A third function is the ability of HA to bind to cell surface receptors and induce intracellular signals, leading to changes in cellular behavior. Cluster determinant 44 (CD44) is considered the principal receptor for HA and is expressed in most cell types (5). Nevertheless, HA can also interact with receptor for hyaluronate-mediated motility (RHAMM) in fibroblasts, smooth muscle cells, macrophages, T lymphocytes, and multiple cancer cells, but the expression of RHAMM is generally low (31). Binding of HA to CD44 or RHAMM can trigger a number of intracellular pathways, including those that involve PKC, focal adhesion kinase (FAK), MAP kinases, NFκB, PI3K, tyrosine kinases and cytoskeletal components (13, 142). Several other HA-binding proteins have been identified in the cell membrane, including lymphatic vessel endothelial HA receptor (LYVE-1), hyaluronan receptor for endocytosis (HARE), toll-like receptor 4 (TLR-4), layilin, and intercellular adhesion molecule 1 (ICAM-1),

however, the expression of these proteins is very low and restricted to specific cell types (12, 63, 87, 115).

As a signal initiator, HA exerts significant effects on cell proliferation, differentiation, migration and invasion and has important repercussions in multiple physiological and pathological events (152, 154). During development, for example, HA participates in the formation of various organs and regions including the brain, heart, limbs and joints via induction of EMT and formation of hydrated matrices that physically separate different cell layers (142). During wound healing, HA accelerates wound closure by stimulating the expression of adhesion molecules and inducing fibroblast proliferation and neovascularization (45). HA also contributes to inflammation by inducing the expression of chemokines in machrophages, which are important for the development and maintenance of the inflammatory response (90). During cancer, HA influences the malignant behavior by interfering with the intercellular adhesion machinery, inducing cell growth and metastasis and promoting tumor angiogenesis (61).

HA is synthesized in vertebrates and in some microorganisms by transmembrane glycosyltransferases known as hyaluronan synthases (Has). These proteins sequentially assemble two distinct monosaccharides, N-acetyl-glucosamine (UDP-GlcNac) and glucuronic acid (UDP-GlcA), in the presence of Mg^{2+} or Mn^{2+} to form large HA polymers without any protein core (164). This is an important difference between HA and other glycosaminoglycans, which are synthesized as relatively small

molecules (15-20 kDa) and covalently attached to core proteins (80). In mammalian organisms, three Has genes have been identified (Has1, Has2 and Has3), which are evolutionarily conserved, although located on different chromosomes. Has1 is found on human chromosome (Chr) 19q13.4 and mouse Chr 17; HAS2 on human Chr 8q24.12 and mouse Chr 15; HAS3 on human Chr 16q22.1 and mouse Chr 8 (141).

The mammalian Has are composed of seven putative membrane-spanning regions; two located at the N-terminal end and five at the C-terminal end of the protein (Fig 1.5). They also contain a large cytoplasmic loop where the glycosyltransferase catalytic sites and UDP-binding motifs are found (60). Despite structural similarities and 50-70% homology between Has1, 2 and 3, these proteins present differences in expression patterns and in the size of HA produced, suggesting specific and independent roles for each of these synthases.

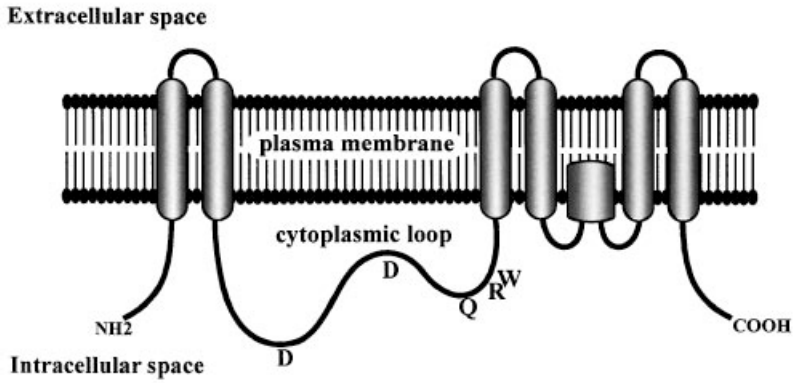

Figure 1.7. Structure of mammalian hyaluronan synthases. All three mammalian Has isoforms contain seven transmembrane domains. The predicted cytoplasmic loop contains aminoacids responsible for glycotransferase activity. Adapted from Itano et al (60).

Throughout development, Has1 is only expressed during gastrulation and early neurulation while Has3 is restricted to expression within the developing teeth and facial hair follicles (142). In contrast, Has2 is widely expressed, with major sites including the developing heart and skeleton and the emergent neural crest (142). The relevance of these high levels of Has2 expression during cardiovascular development has been established through the use of gene knock out technology. Mouse embryos lacking the Has2 gene exhibit severe cardiovascular defects such as failure to form the endocardial cushions and absence of an organized vascular network (18). These abnormalities are due to abrogation of HA production and subsequent impairment of the EMT process and lead to death by embryonic day (E) 9.5.

HA is synthesized by Has2 as a high molecular weight polysaccharide of 1-2 Mega Daltons. In addition, HA can be degraded via exo-glycosidases and hyaluronidases during local or systemic turnover of the carbohydrate and, as a consequence, multiple sizes of HA may coexist (Figure 1.8) (144). These differences in molecular mass have been recognized as an important factor governing the ability of HA to alter cell behavior, although there is no agreement in regards to which HA type is relevant for the regulation of EMT processes. In studies of disease states such as cancer, for example, it has been shown that native HA, but not HA fragments, induces migration of melanoma and breast cancer cells (13, 74). On the other hand, studies in Lewis lung carcinoma (3LL) cells have yielded opposing results, with HA olygomers being responsible for cellular motility (39). Similar conflicting effects of different HA sizes have been reported in embryonic cells. While HA fragments

promote the migration of primary embryonic fibroblasts, it is the high molecular weight HA, and not the smaller HA fragments, that induces stem cell differentiation and endocardial cushion EMT (32, 39, 123). Thus, there is an imperative need for additional studies that will answer crucial questions in regards to the relationship between HA size and its biological activity. Furthermore, the specific mechanisms that govern Has2-mediated production of HA and HA-stimulated cellular responses during embryogenesis remain unclear. Therefore, this dissertation focuses on defining and understanding these important molecular interactions.

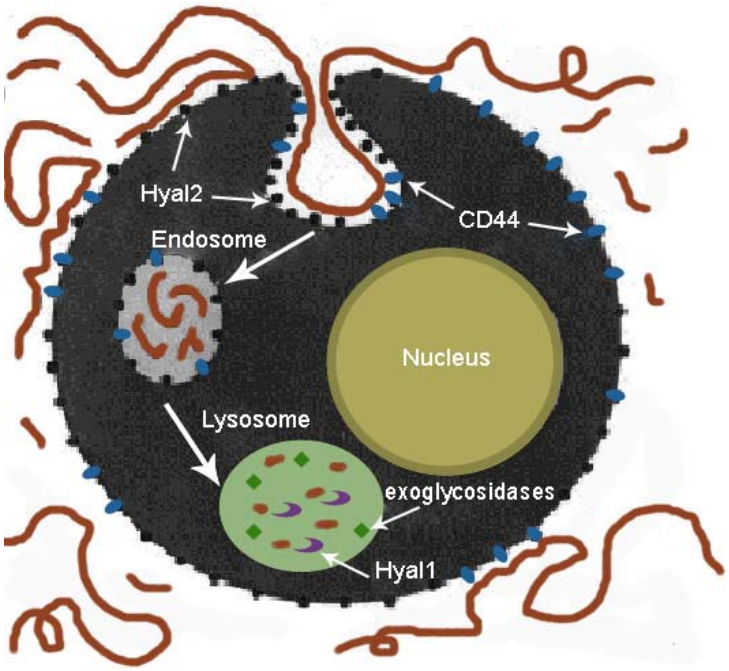

Figure 1.8. Mechanisms for HA degradation. Membrane hyaluronidases (Hyal2), in cooperation with HA receptors such as CD44, cleave high molecular weight HA polymers into smaller fragments (20-40 kDa). These fragments can be released into the extracellular space or they can be subjected to endocytosis and further degradation by lysosomal hyaluronidases (Hyal1) and exoglycosidases.

1.4. Hypothesis

The objective of our studies was to identify key signaling molecules for the regulation of Has2 and the induction of HA-mediated biological activity as it is relevant for cardiogenesis. Although few studies have been carried out regarding HA signal transduction mechanisms in the context of development, research in cancer cells suggests that HA may be able to activate intracellular kinases and transcription factors to promote EMT and invasive cellular responses. Furthermore, growth factors have been shown to induce Has2 phosphorylation in normal adult cells and cancer models. This phosphorylation is thought to be important for Has2 activation and subsequent synthesis of HA. However, no functional connection has been established between growth factors and their potential to regulate HA production and signals during embryogenesis. Thus, **we hypothesized that Has2 increases HA production in response to TGFβ2 and EGF leading to epithelial to mesenchymal transition (EMT) and cellular invasion during cardiovascular development.**

CHAPTER 2

HYALURONAN INDUCES DIFFERENTIATION AND INVASION OF EMBRYONIC CELLS IN A SIZE DEPENDENT MANNER

This chapter has been adapted from the following publications:

Craig EA, Parker P, Camenisch TD. Size-dependent regulation of Snail2 by hyaluronan: its role in cellular invasion. Glycobiology. 2009 Aug; 19(8):890-8. Epub 2009 May 18.

Craig EA, Parker P, Austin AF, Barnett JV, Camenisch TD. Involvement of the MEKK1 signaling pathway in the regulation of epicardial cell behavior by hyaluronan. Cell Signal. 2010 Jun;22(6):968-76. Epub 2010 Feb 14.

2.1. Introduction

Hyaluronan or hyaluronic acid (HA) is a linear carbohydrate comprised of repeating disaccharide units of glucuronic acid and N-acetylglucosamine. HA belongs to the glycosaminoglycan family, which also includes heparan sulfate, chondroitin sulphate, dermatan sulphate and heparin (44). This relatively simple molecule is a critical structural component of the extracellular matrix (ECM) and also acts as a signaling initiator, thus eliciting significant changes in cellular behavior. A wealth of research has identified roles for HA in multiple processes including cell adhesion,

proliferation, invasion, differentiation, EMT and angiogenesis under normal as well as pathological conditions.

In vertebrates, HA is produced at the inner face of the plasma membrane by hyaluronan synthases and is exported into the extracellular space as a high molecular mass polymer (~$1x10^6$ Da) (101, 108). Once outside the cell, HA can be enzymatically degraded through the coordinated activity of hyaluronidases, and two exoglycosidases (beta-glucuronidase and beta-N-acetyl-glucosaminidase) that remove the terminal sugars (144). In mammals, the hyaluronidase family can be found in two gene clusters that express six related proteins: Hyal1, Hyal2, Hyal3, Hyal4, HyalP1 and PH-20. However, only Hyal1 and Hyal2 are highly expressed in somatic cells and as such, they are primarily responsible for the degradation of HA (26). The major site of action for Hyal1 is the lysosome while Hyal2 is commonly found at the cell surface, although it can also be present within the lysosome. Cleaved HA oligosaccharides have been shown to exert distinct biological activity from that of their high molecular weight counterparts and in some cases they can even act as antagonists to the native HA (142, 165, 166). This functional difference between HAs of varying molecular masses is a matter of controversy as many studies show opposing results in regards to which type of HA can bring about cellular changes (74, 89). These discrepancies may be due to differences in experimental settings, purity of HA reagents and the possibility of diverse responses to HA depending on the cell type (88).

In the developing heart, HA is highly abundant around tissues where EMT occurs such as the epicardium and the areas surrounding the endocardial cushions. Mice lacking hyaluronan synthase 2 (Has2), the primary enzyme responsible for the production of HA during development, contain very little HA, fail to undergo endocardial cushion EMT, and as a result exhibit severe cardiovascular defects, leading to death by embryonic day E 9.5. Furthermore, endocardial cushion explants from $Has2^{-/-}$ mice can be stimulated to undergo EMT by addition of exogenous high molecular weight HA, thus suggesting that native HA is crucial for embryogenesis (18). However, because these HA-deficient mice die before the formation of the epicardium at around day E10.5 (76), it is not known whether HA also plays a role in later developmental events such as epicardial EMT. Furthermore, other studies suggest that HA fragments also stimulate the migration of embryonic cells, thus the specific HA size that may be relevant for the induction of responses in embryonic cells remains unclear (39).

This chapter addresses our first aim, which was to investigate the role of hyaluronan size in the induction of biological activity in embryonic cells. With this aim we wanted to: a) Compare and contrast the effect of high versus low molecular weight hyaluronan in the induction of cellular invasion in epithelial and mesenchymal embryonic cells. b) Determine whether hyaluronan also plays a role in other EMT steps such as proliferation and differentiation.

For this purpose we used two different HA sizes, one representing the initial high molecular weight at which HA is produced by Has2 (~1 MDa) and another much smaller type of HA (~31 kDa), which is similar in size to HA fragments resulting from membrane hyaluronidase degradation (26, 101). This allowed us to determine whether HA must be cleaved in order to induce cell signals during development.

We employed two different cell lines to assess whether HA differentially affects cells at various stages of EMT. Our epicardial cell line, which is epithelial in phenotype, allowed us to explore the role of HA in early EMT steps, such as proliferation and differentiation. On the other hand, our NIH-3T3 cell line, which is mesenchymal in phenotype, provided us with a model to study whether HA has any effect in embryonic cells that are already differentiated.

2.2. Results

2.2.1. HMW-HA promotes cellular invasion but not proliferation:

As cells can either “go or grow” but can not perform both cellular functions at the same time, we sought to determine whether HA instructs embryonic cells to invade or proliferate. Cell growth in response to HA was assessed 24 hours post-treatment using the MTT proliferation assay. As shown in Figure 2.1A, NIH-3T3 fibroblasts as well as epicardial cells treated with LMW-HA or HMW-HA showed no difference in growth as compared to the untreated controls. These results demonstrate that, regardless of molecular weight, HA does not affect embryonic cell proliferation.

To study the effect of HA on cellular invasion, we performed an in vitro three-dimensional invasion assay based on a modified Boyden chamber assay. Briefly, in a two-chamber system, collagen gels were cast in the upper chamber over an 8□m pore membrane while the bottom chamber was filled with 20% FBS to induce chemotaxis. Cells labeled with CalceinAM were placed on top of the gel, pulse treated with different HA sizes for 30 minutes and allowed to invade for 24 hours. While cells treated with LMW-HA showed responses similar to the untreated controls, cells stimulated with HMW-HA exhibited a significant increase in their invasive response (Figure 2.1B). These results show that HMW-HA, but not the smaller LMW-HA, promotes cellular invasion.

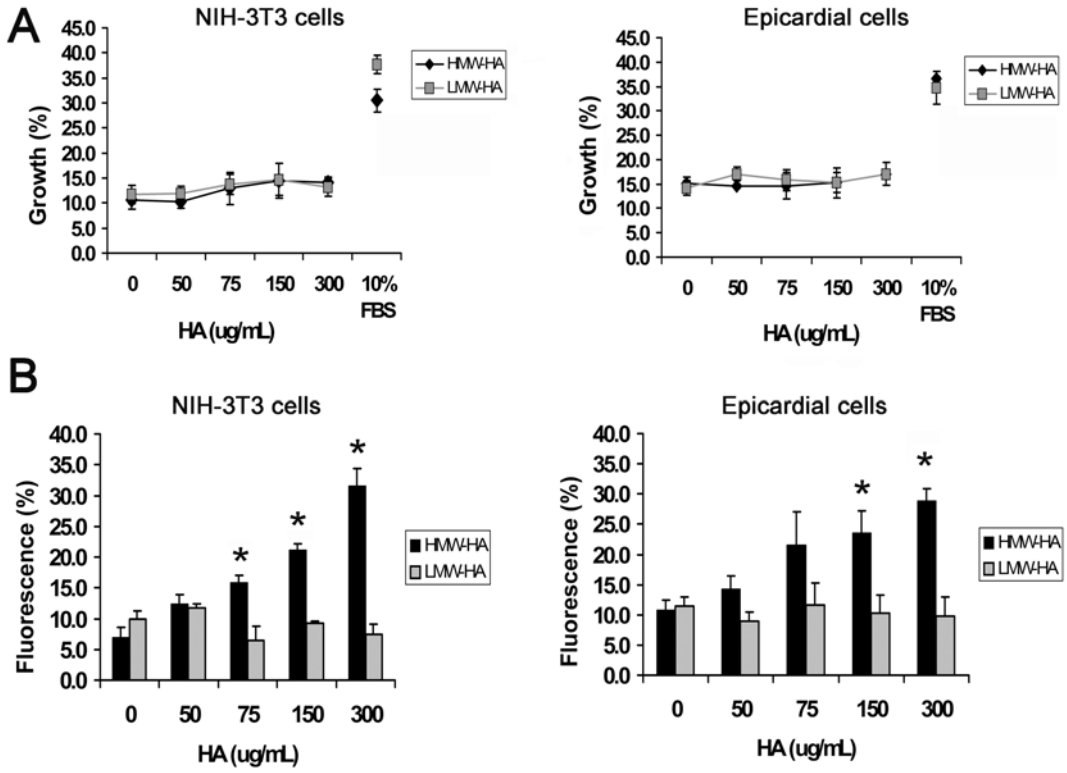

Figure 2.1. Effect of HA size on embryonic cell proliferation and invasion. (A) Cells were treated with LMW-HA or HMW-HA for 30 minutes and incubated for 24 hours in low serum medium (1% FBS). Cell proliferation was assessed using the MTT

assay. 10% FBS was used as a positive control. (B) Fluorescently labeled cells were stimulated with LMW-HA or HMW-HA for 30 minutes and incubated for 24 hours to allow for invasion into collagen gels and through an 8□m pore membrane. *, $p<0.05$ as compared to the untreated control.

2.2.2. HMW-HA but not LMW-HA induces differentiation of epicardial cells:

Because NIH-3T3 cells are already differentiated, we assessed the role of HA size on cellular differentiation only in epicardial cells, which have an epithelial phenotype and thus can further undergo morphological changes. For these studies, we performed Western Blots to determine whether HA had any effect on vimentin protein levels. Vimentin is an intermediate filament protein that is highly expressed in cells that have differentiated into a mesenchymal phenotype but is of low abundance in most epithelial cells. Thus, vimentin constitutes a robust mesenchymal cell marker.

Stimulation of epicardial cells with HMW-HA for 30 minutes, followed by a 24 hr incubation period, significantly increases the expression of vimentin in a dose-dependent manner (Fig. 2.2). However, cells treated with similar doses of LMW-HA did not show any changes in vimentin protein levels. These results demonstrate that HA size is important for its biological activity and that only the native HMW-HA is able to regulate embryonic cell behavior. Furthermore, HA appears to regulate cellular responses, both during early stages of EMT, such as differentiation, as well as during the later stages involving cellular invasion.

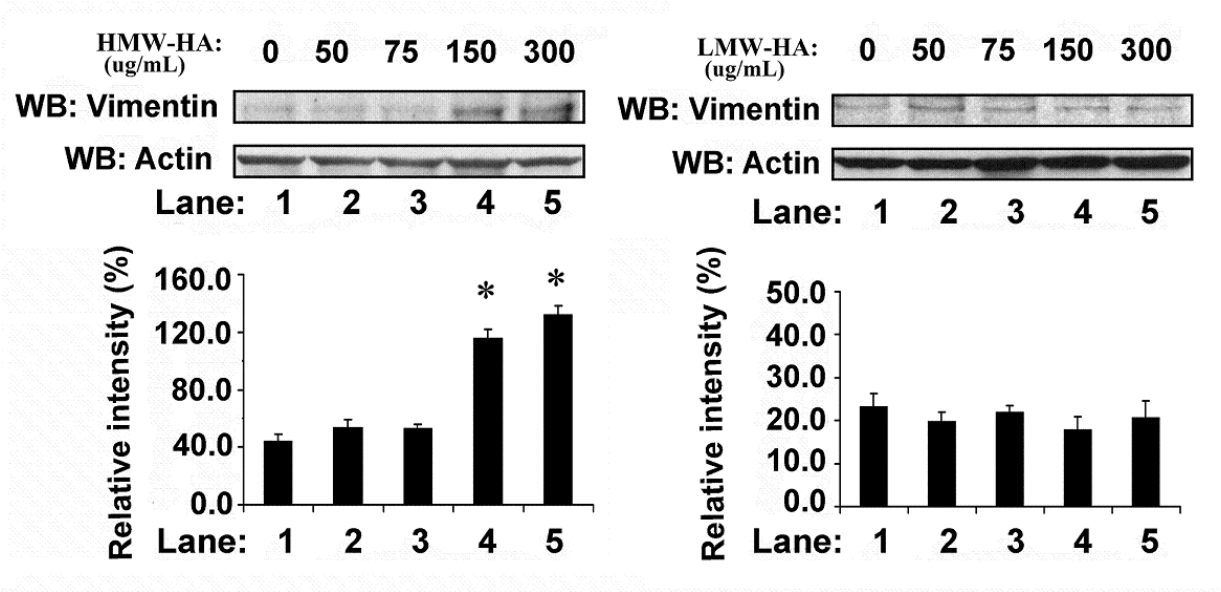

Figure 2.2. Role of HA on epicardial cell differentiation. Cells were treated with HMW-HA or LMW-HA for 30 minutes and incubated for 24 hours. Lysates were subjected to WB with anti-vimentin and anti-actin antibodies. The graphs represent WB quantification by densitometry. The results are expressed as vimentin band intensity relative to the actin bands. *, $p<0.05$ as compared to the untreated control.

2.3. Discussion

HA is present in high levels around cells undergoing migration and invasion during embryonic morphogenesis, e.g, around neural crest cells migrating to form peripheral ganglia (107, 113), around mesenchymal cells invading the primary corneal stroma (75) and around cardiac mesenchyme traveling into endocardial cushions during heart valve formation (134). Lack of HA in these areas leads to severe developmental abnormalities, suggesting an important role for HA during embryogenesis.

Independent studies have implicated HA in the malignant EMT process, cell proliferation and migration using in vivo approaches as well as cancer cell lines (14, 105). On the other hand, experiments utilizing embryonic cells such as NIH-3T3s have yielded controversial results that may however be explained by differences in HA concentrations. While experiments using HMW-HA doses as low as 10 μg/mL have failed to detect any biological response in NIH-3T3 cells (155), other studies employing the same cell line have reported significant changes in cellular behavior by treating with HMW-HA concentrations as high as 1000 μg/mL (95). Thus, to obtain more accurate results in our experiments we used HA doses between 50 and 300 μg/mL, which more closely resemble normal HA concentrations during development (143). We also compared and contrasted the effects of HMW-HA versus LMW-HA as many conflicting results have been published in regards to which molecular size is more relevant in the context of development.

In the present study we demonstrate that both HMW-HA and LMW-HA have negligible effects on embryonic cell proliferation at normal physiological concentrations. On the other hand, HMW-HA is able to induce invasion in both cell lines, although a slightly higher response is observed in NIH-3T3 cells. These differential effects may be explained by the morphological differences present in the two cells lines employed, with NIH-3T3 being mesenchymal while epicardial cells are epithelial in phenotype. HMW-HA also induces differentiation of epicardial cells, thus it is likely that these cells must differentiate in order to acquire increased motility in response to HA.

These results demonstrate that HA activity is dependent on its molecular size and that embryonic cells are only responsive to HMW-HA. This adds to the growing evidence suggesting that native HA is specifically important for the induction of signals in embryonic as well as in adult cardiac cells (18, 171). Furthermore, these findings highlight a novel HA-mediated response in the epicardium and indicate a contribution for this molecule in the formation of the coronary vasculature.

2.4. Experimental Procedures

Cell culture:

NIH-3T3 mouse fibroblasts were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM containing 10% FBS and 1% antimycotic/antibiotic solution (penicillin, streptomycin and amphotericin) (Mediatech Inc.). NIH-3T3 cell cultures were maintained in an atmosphere of 5% CO_2 at 37°C in a humidified incubator. The inducible immortalized epicardial cell line was generated as previously described (6). Briefly, embryonic hearts from transgenic mice harboring a thermolabile SV40 TAg gene were harvested at E11.5 and placed dorsal side down on collagen culture dishes. Hearts were then incubated for 15 hrs at 37°C to allow for epicardial monolayers to form at the collagen coated surface. The resulting monolayers were propagated at 33°C in Dulbecco's modified Eagle´s medium (DMEM) containing 10% fetal bovine serum (FBS), antibiotics, insulin-transerrin-selenium (Invitrogen, Carlsbad, CA) and mouse gamma interferon (10 units/mL, R&D systems, Minneapolis, MN).

Hyaluronan:

High molecular weight hyaluronan (HA) (900- 1,200 kDa; average mass of 980 kDa; catalog #GLR002) and low molecular weight hyaluronan (15- 40 kDa; average mass of 31 kDa; catalog #GLR001) were obtained from R&D systems. Stock solutions were prepared by reconstituting HA in sterile water, boiling for 30 minutes at 100°C to inactivate any contaminants, and addition of DMEM.

Western Blotting:

Total cellular lysates were prepared from cells and subjected to sodium docecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electroblot transfer onto polyvinylidene difluoride membrane and blocking in 3% nonfat milk, primary antibodies against vimentin(Cell Signaling Technology, Danvers, MA), or β-actin (ABR, Golden, CO) were used followed by secondary antibodies. Detection was performed using Super Signal West Pico substrate (Pierce, Rockford, IL). Western blot images were quantified using the Image J software (NIH, Bethesda, MD).

Proliferation Assays:

Cells were seeded into 96-well culture plates at a density of 1.8×10^4 cells/well and routinely cultured overnight. Next, cells were serum starved for 1 hour, followed by 30 minute treatment with HA at 0-300 μg/mL and a 24 hour incubation period. Cell proliferation was assessed using the Vybrant® MTT cell proliferation assay kit (Molecular Probes) as indicated by the manufacturer.

Invasion Assays:

The invasive potential of mouse embryonic cells upon HA stimulation was determined by using a transwell chamber system with 8μm pore polyester membrane inserts (Corning Inc., Corning, NY). Collagen was neutralized to pH 7.4 with a buffer containing 10X M199 and 2.2% sodium bicarbonate. The collagen was allowed to polymerize on top of the membrane at room temperature for 30 min. Next, DMEM+20% FBS was added to the lower chamber as a chemoattractant. Cells were fluorescently labeled with CalceinAM (BD Biosciences) and plated on top of the collagen layer at a density of 2.0×10^4 cells per insert in serum-free DMEM. After 30 minute treatment with HA at 50-300 μg/ml, cells were washed with PBS and incubated in serum free DMEM for 24 hours. Following incubation, transwell inserts were removed from the plate containing 20% FBS in DMEM and positioned in a plate with 2mM EDTA in PBS for 15 minutes. Invasion was quantified by measuring fluorescently labeled cells that crossed the polyester membrane and were detached into the EDTA solution. Fluorescence was determined with a plate reader at 538 nm (Spectramax Gemini, Molecular Devices, Sunnyvale, CA).

Statistical Analysis:

Quantitative data were analyzed using two sample Student's t tests. The quantitative data displayed represent the means ± S.D. of 2 independent experiments performed in triplicate, except for invasion assays, in which samples were analyzed in sets of 6. The level of significance was established at $P<0.05$.

CHAPTER 3

CD44 AND MEKK1 DEPENDENT PATHWAYS PLAY AN IMPORTANT ROLE IN THE TRANSDUCTION OF HYALURONAN SIGNALS DURING DEVELOPMENT

Portions of this chapter have been adapted from the following publications:

Craig EA, Parker P, Camenisch TD. Size-dependent regulation of Snail2 by hyaluronan: its role in cellular invasion. Glycobiology. 2009 Aug; 19(8):890-8. Epub 2009 May 18.

Craig EA, Parker P, Austin AF, Barnett JV, Camenisch TD. Involvement of the MEKK1 signaling pathway in the regulation of epicardial cell behavior by hyaluronan. Cell Signal. 2010 Jun;22(6):968-76. Epub 2010 Feb 14.

3.1. Introduction

Despite its simple carbohydrate structure, HA performs multiple physiological roles and is crucial for tissue formation, maintenance and remodeling. HA not only provides structural support as a component of the extracellular matrix, but also is able to influence morphogenetic processes by directly regulating cellular responses. HA has been shown to initiate signals by binding to cell surface receptors such as CD44 (13), RHAMM (50) and LYVE-1 (115), with CD44 being the most widely expressed (5). Interactions between HA and CD44 (5) can lead to the activation of intracellular signaling pathways which mediate changes in cell morphology, proliferation, migration

and invasion (14, 105). These effects on cellular behavior play a crucial role during normal developmental processes as well as during cancer progression.

CD44 is a single spanning transmembrane glycoprotein that is expressed in a wide variety of tissues (131). The CD44 gene contains 20 exons, 10 of which can be alternatively spliced to give rise to many CD44 isoforms which bind HA with different affinities (36, 82). The most common CD44 isoform is the CD44 standard (CD44s) and is composed of a large extracellular domain of 248 amino acids, a 21 amino acid membrane spanning segment and a relatively short cytoplasmic portion of 72 amino acids. Other CD44 variants (CD44v) are created by insertion of varying aminoacids at the membrane proximal region of the extracellular domain but their expression is highly restricted to specific tissues (84). The ability of CD44 to bind HA appears to be regulated not only by structural variations in the CD44 extracellular domain but also by post-translational modifications such as phosphorylation, sulphation, and glycosylation (8, 99). Thus, the regulation of the amount and type of postranslational modification can add further diversity to the range of potential functions of CD44/HA interactions.

CD44 regulates cell shape and motility by physically mediating the interaction of the ECM with the intracellular actin cytoskeleton (112). However, CD44 may also transduce HA signals by activating a number of protein kinases and transcription factors, depending on the cellular context. One of the proposed signaling effectors following HA/CD44 interaction in cancer cells is the transcriptional regulator nuclear

factor kappa B (NFκB) (74). However, it remains to be elucidated whether HA can activate NFκB during normal physiological processes such as embryonic development. NFκB is involved in cell survival, immune function, epithelial to mesenchymal transition (EMT) and cellular invasion (7, 14, 158, 168). This transcription factor is present in the cytosol complexed to an inhibitory kappa B (IκB) monomer. Signals that induce NFκB activity cause the phosphorylation of IκB and subsequent dissociation of the inhibitory complex thereby allowing free NFκB to translocate to the nucleus and activate target genes. Elevated NFκB expression has been associated with colon and breast cancer progression (57, 137). Additionally, inhibition of NFκB during early embryonic development leads to the abnormal formation of cardiac structures such as the valves and septum (55). Furthermore, although the mechanisms by which NFκB becomes activated during development are poorly understood, overexpression of NFκB in frog embryos has been shown to increase the transcription of many genes, including Snail family members (173).

Other intracellular molecules that may mediate HA signals include c-Src kinase (15), PI3K (2), and the extracellular signal regulated kinases 1 and 2 (ERK1/2) (13). Nevertheless, the exact mechanism through which HA transduces its signals from the plasma membrane remains unclear, as HA receptors do not possess kinase activity, and thus are not able to directly phosphorylate downstream proteins. Coincidentally, several of these HA-activated molecules, namely NFκB and ERK1/2, are highly expressed in the developing heart (21, 55) and are induced in migratory cells by

members of the mitogen-activated protein kinase (MAPK) cascade, such as MEK kinase 1 (MEKK1) (27, 86).

Although mice lacking MEKK1 do not exhibit life-threatening congenital defects, MEKK1 disruption results in the impairment of embryonic epithelial cell motility both in vivo and in vitro (169, 172), suggesting that MEKK1 is an important contributor in the regulation of epithelial cell biology during development.

This chapter addresses our second aim, which was to determine the signaling mechanisms by which hyaluronan promotes changes in cellular behavior. Here our goal was to: a) Determine the role of the receptor CD44 in hyaluronan-mediated cellular responses. b) Identify molecules that may interact with CD44 in response to hyaluronan to induce downstream signaling. c) Elucidate the contribution of the ERK and NFκB pathways in the transduction of hyaluronan signals to promote EMT and cellular invasion.

For this part of our studies, we again employed our epithelial epicardial cell line and the mesenchymal NIH-3T3 cell line to examine whether HA similarly or differentially activates signaling pathways in embryonic cells, depending on their phenotype. We specifically focused on the role of the receptor CD44 in HA-mediated cellular responses because of its high expression in the heart and explored the possible contribution of MEKK1-dependent pathways in the transduction of HA signals (72). Our hypothesis is that HMW-HA induces CD44/MEKK1 association and

activation of downstream signals leading to embryonic cell differentiation and invasion. In order to identify key intracellular players, we employed a variety of pharmacological inhibitors and specific siRNAs coupled with techniques described in section 3.4.

3.2. Results

3.2.1. HMW-HA induces CD44-MEKK1 interaction and activation of MEKK1:

Although the activation of MEKK1 and interactions between HA and the CD44 receptor have been shown to play an important role in epithelial and mesenchymal cell motility, no functional connection between these molecules has been reported to date (34, 48, 73). Thus, we sought to determine whether MEKK1 physically associates with CD44 in embryonic cells following HA stimulation. To address this question, we treated our NIH-3T3 and epicardial cell lines with 300 μg/mL of HMW-HA for various time points (0-15 min) followed by immunoprecipitation with an antibody against CD44. Subsequent Western blotting was performed to detect MEKK1 and CD44. As shown in Figure 3.1.A, stimulation with HMW-HA for 5 minutes induces recruitment of MEKK1 to CD44 in both cell types. Cells treated with LMW-HA or pretreated with the CD44 blocking antibody KM201 followed by stimulation with HMW-HA fail to show CD44/MEKK1 association (Fig. 3.1 B). This suggests that HMW-HA physically binds CD44 in order to induce the interaction of this receptor with MEKK1. Furthermore, this interaction appears to occur, regardless of the cellular phenotype as a similar response was observed in both NIH-3T3 and epicardial cells.

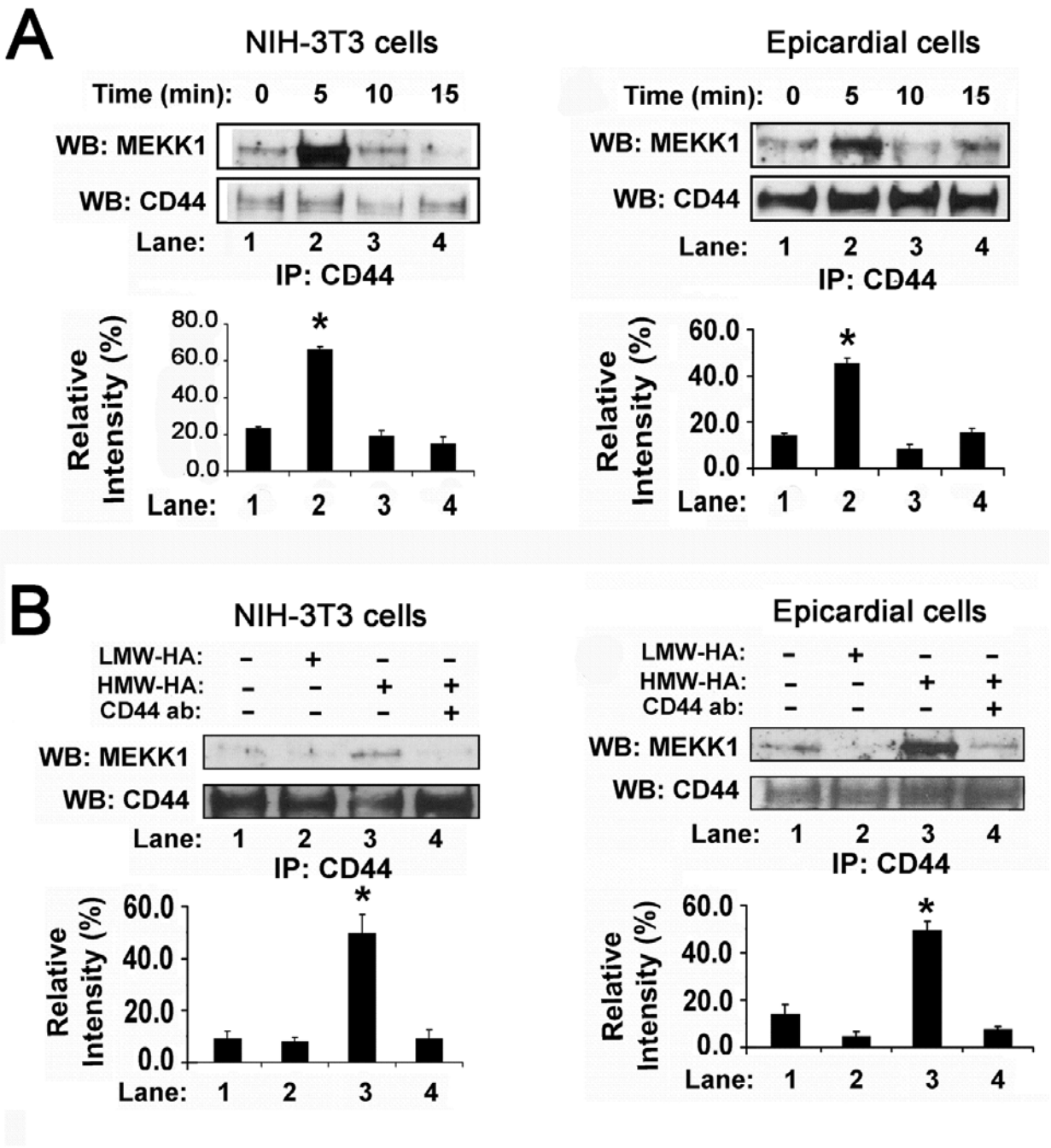

Figure 3.1. MEKK1 association with CD44 in response to HMW-HA. (A) Cells were treated with HMW-HA (300 μg/mL) for 0-15 min, followed by immunoprecipitation (IP) with anti-CD44 and Western blotting (WB) with anti-MEKK1 (top panel) or anti-CD44 antibody (bottom panel). The graphs represent WB quantification by densitometry. The results are expressed as MEKK1 band intensity relative to the CD44 bands. (B) Cells were left untreated (lane 1), treated with LMW-HA (lane 2) or HMW-HA (lane 3) for 5 min or incubated with CD44 blocking antibody followed by treatment with HMW-HA for 5 min (lane 4). Lysates were subjected to IP

with anti-CD44 and WB with anti-MEKK1 (top panel) or anti-CD44 antibody (bottom panel). Densitometry analysis was performed as in 3.1A. *, p<0.05 as compared to the untreated control.

We next assessed whether HMW-HA plays a role in the activation of MEKK1 by determining the phosphorylation state of this protein at various time points following HA treatments. As illustrated in Figure 3.2, cells stimulated with HMW-HA show increased phosphorylation of MEKK1 on threonine 1383, which is located within the kinase domain of this protein. This phosphorylation is observed after 10 and 15 minutes in NIH-3T3 cells, while in epicardial cells it occurs after only 5 minutes. This indicates that the spacio-temporal regulation of MEKK1 activity by HA is dependent on the cell type. Furthermore, these findings suggest that association of MEKK1 with CD44 in response to HA results in the activation of MEKK1.

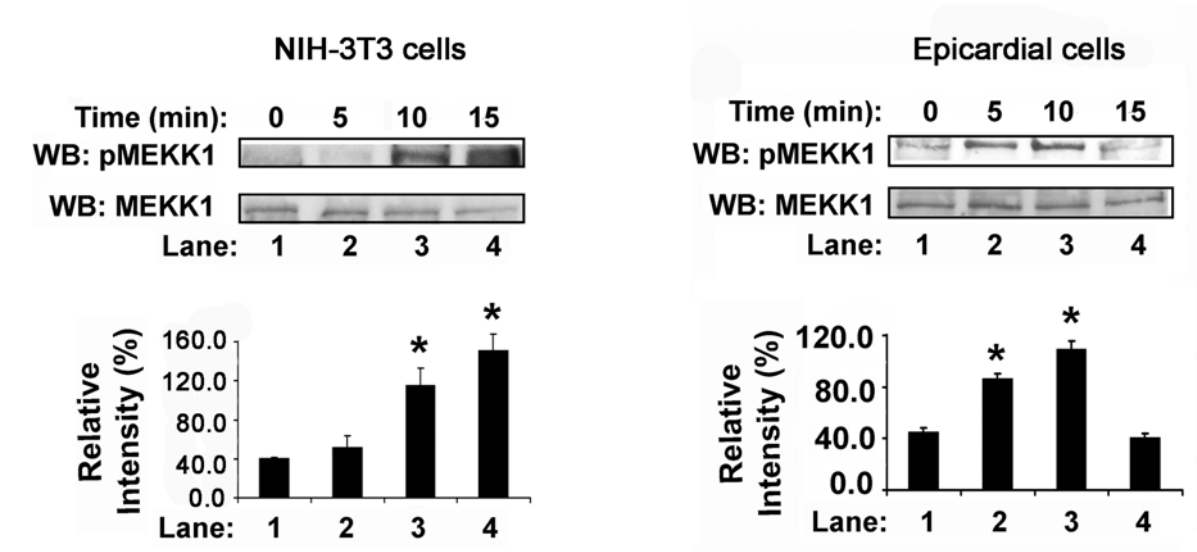

Figure 3.2. HMW-HA induces MEKK1 phosphorylation. Cells were treated with HMW-HA (300 μg/mL) for 0-15 min, followed by WB with anti-phospho MEKK1 (top panel) and reblotting with anti-MEKK1 (bottom panel). Densitometry measurements were performed as in fig. 3.1 and are expressed as phospho-MEKK1 band intensity relative to the MEKK1 bands. *, p<0.05 as compared to the untreated control.

3.2.2. HMW-HA promotes ERK phosphorylation and SRE activation via CD44 and MEKK1:

Both HA and MEKK1 have been involved in the activation of the ERK1/2 signaling pathway during cancer metastasis (13, 146). However, it is not known whether these molecules also contribute to the regulation of cellular responses during development. Therefore, we investigated whether ERK1/2 is activated in embryonic cells following stimulation with HMW-HA and whether CD44 and MEKK1 play a role in the transduction of these signals.

ERK1/2 phosphorylation status in response to various HA concentrations was determined via Western blotting and a fast activated cell enzyme-linked immunosorbent assay (FACE). We found that HMW-HA significantly stimulates the phosphorylation of ERK1/2 in a dose-dependent manner starting at 150 μg/mL of HA (Fig. 3.3A). However, treatment with LMW-HA does not affect ERK1/2 phosphorylation status, indicating specific response to the HMW-HA size (Fig. 3.3B). Furthermore, pretreatment with CD44 blocking antibody or transfection with KI-MEKK1, followed by stimulation with HMW-HA significantly decreases ERK1/2 phosphorylation as compared to treatments with HMW-HA alone (Fig. 3.3B). These data indicate that CD44 and MEKK1 are important for the activation of ERK1/2 by HMW-HA, although other molecules may also be involved. Interestingly, ERK1/2 phosphorylation appears to occur exclusively through the CD44 receptor in NIH-3T3 cells, as blockade of CD44 completely abrogates the ability of HA to induce ERK1/2 activation. On the other hand, CD44 is only partially necessary for ERK1/2

phosphorylation in epicardial cells, indicating the possible involvement of other HA receptors in this response.

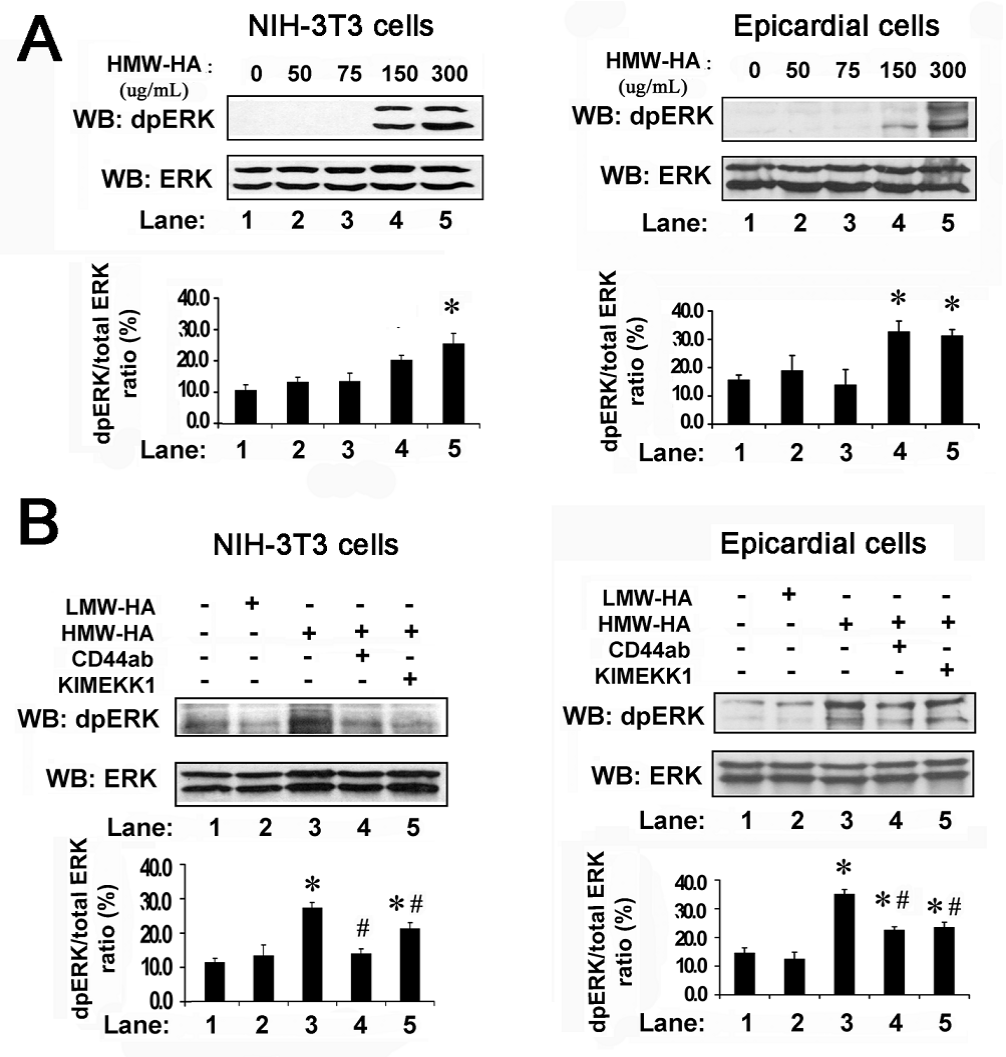

Figure 3.3. Mechanisms of HA-mediated ERK phosphorylation. (A) Cells treated with HMW-HA (0-300 μg/mL) for 15 min were employed for WB with anti-diphospho-ERK1/2 (top panel) and reblotted with anti-ERK1/2 (bottom panel) or were subjected to FACE assays (graph) and the absorbance values were measured. (B) Cells were left untreated (lane 1), or stimulated with LMW-HA (lane 2), HMW-HA (lane 3), HMW-HA in the presence of a CD44 blocking antibody (lane 4) or HMW-HA in the presence of KI-MEKK1 (lane 5). Lysates were employed for WB with anti-diphospho-ERK1/2 (top panel) and reblotted with anti-ERK1/2 (bottom panel) or cells were subjected to FACE assays (graph) and the absorbance values were measured. All HA treatments were performed for 15 min. *, $p < 0.05$ as compared to the untreated control; #, $p < 0.05$ as compared to treatment with HMW-HA alone.

One of the mechanisms through which ERK is known to collaborate in the regulation of cell motility is by increasing gene transcription under the control of the serum response element (SRE) (46). Thus, we examined whether HA-induced ERK1/2 activation could be correlated with increased SRE activity. For this, we transfected cells with a reporter construct consisting of serum embryonic alkaline phosphatase (SEAP) driven by three copies of the SRE consensus sequence. As shown in Figure 3.4A, stimulation with HMW-HA induces a significant activation of the SRE, which is coincident with the reported ERK1/2 phosphorylation pattern. This induction of SRE activity is not observed in the presence of LMW-HA and is greatly decreased when cells are treated with a CD44 blocking antibody, an ERK inhibitor peptide or transfected with KI-MEKK1 previous to the addition of HMW-HA (Fig. 3.4B). These results demonstrate that HMW-HA induces a CD44/MEKK1/ERK cascade to regulate SRE activity. Furthermore, despite this pathway being activated in both NIH-3T3 and epicardial cells, ERK phosphorylation and SRE activation in response to HMW-HA appears more prominent in epicardial cells. This suggests that intracellular pathways may respond differently to HA, based on the specific cellular phenotype.

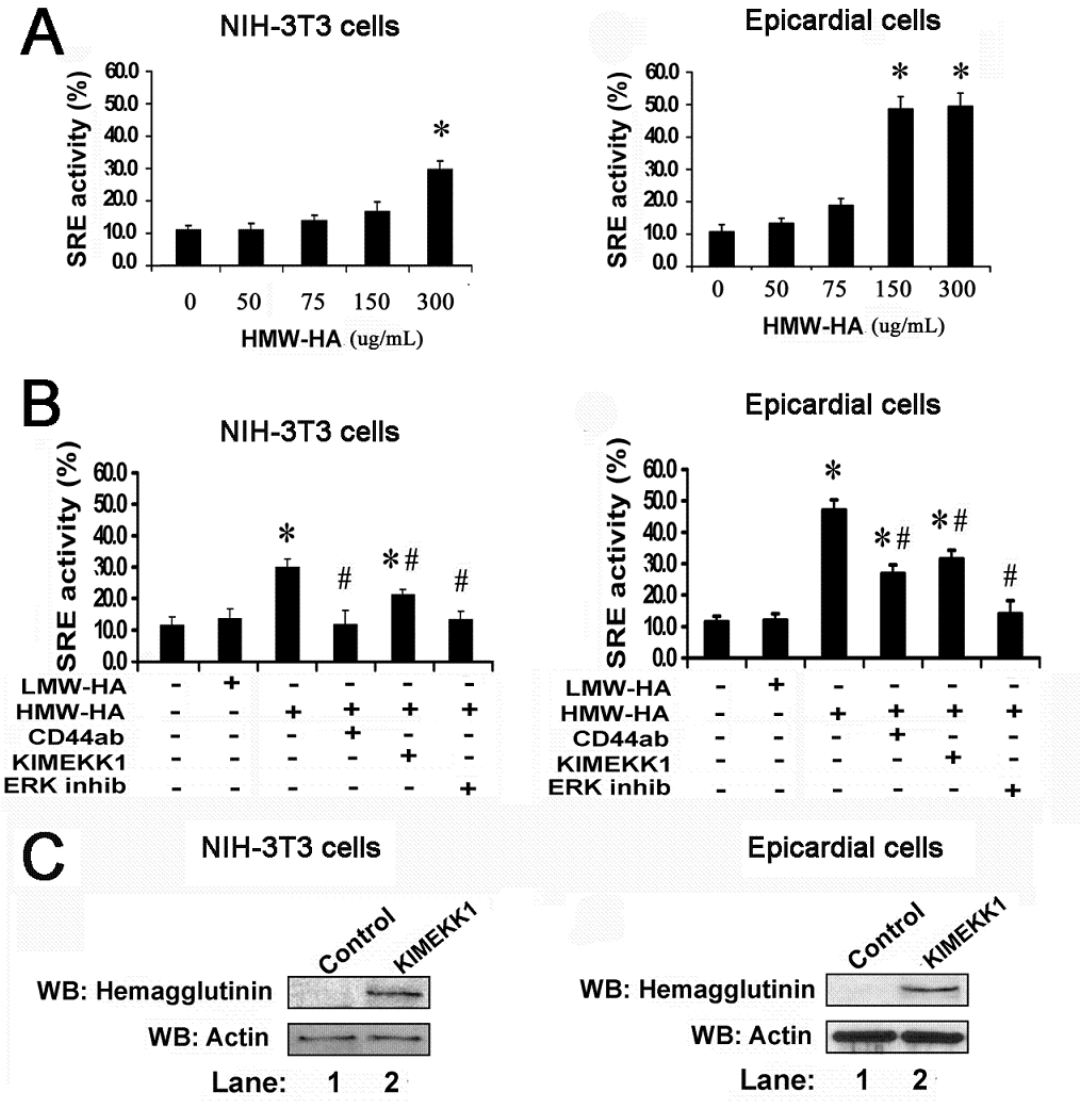

Figure 3.4. Role of CD44 and MEKK1 in the induction of SRE activity by HMW-HA. (A) Cells were transfected with a SEAP-SRE reporter plasmid and treated with HMW-HA for 30 min. SEAP activity was determined after a 24 hr incubation period. (B) Cell were transfected with SEAP-SRE and left untreated or treated with LMW-HA, HMW-HA alone, HMW-HA in the presence of CD44 blocking antibody, HMW-HA in the presence of KI-MEKK1 or HMW-HA in cells incubated with ERK inhibitor peptide. SEAP activity was determined after a 24 hr incubation period. (C) Cells were left untransfected or transfected with KI-MEKK1 and incubated for 30 hrs. Lysates were subjected to WB with anti-hemagglutinin and anti-actin antibodies. *, $p<0.05$ as compared to the untreated control; #, $p<0.05$ as compared to treatment with HMW-HA alone.

3.2.3. HMW-HA regulates NFκB activity and Snail2 expression in a CD44 and MEKK1-dependent but ERK-independent manner:

HA has been shown to induce the activation of the transcription factor NFκB in cancer cells (74). Thus, we investigated whether this protein is also activated in embryonic cells. NFκB activity was assessed by transfecting cells with a NFκB-SEAP reporter construct and measuring SEAP secretion after stimulation with HA. As shown in Figure 3.5A, HMW-HA significantly induces NFκB activity in both cell lines in a dose-dependent manner, with this response being more robust in NIH-3T3 cells. These results were verified by preparing nuclear extracts from cells stimulated with HA and performing Western blots to detect NFκB. Figure 3.5B shows that HMW-HA, but not LMW-HA, induces NFκB translocation into the nucleus and that the NFκB inhibitor SN50 successfully blocks this response.

Next, NFκB-SEAP reporter assays were performed in the presence of specific inhibitors to CD44, MEKK1, and ERK1/2 to determine the role of these molecules in the activation of NFκB by HMW-HA. While pretreatment with CD44 blocking antibody or expression of KI-MEKK1 significantly abrogates the ability of HMW-HA to activate NFκB, incubation with an ERK inhibitor does not appear to disrupt these HA-mediated signals (Fig. 3.5C). These observations suggest that HMW-HA induces NFκB activity through CD44 and MEKK1 in a manner that is independent of ERK1/2 activation.

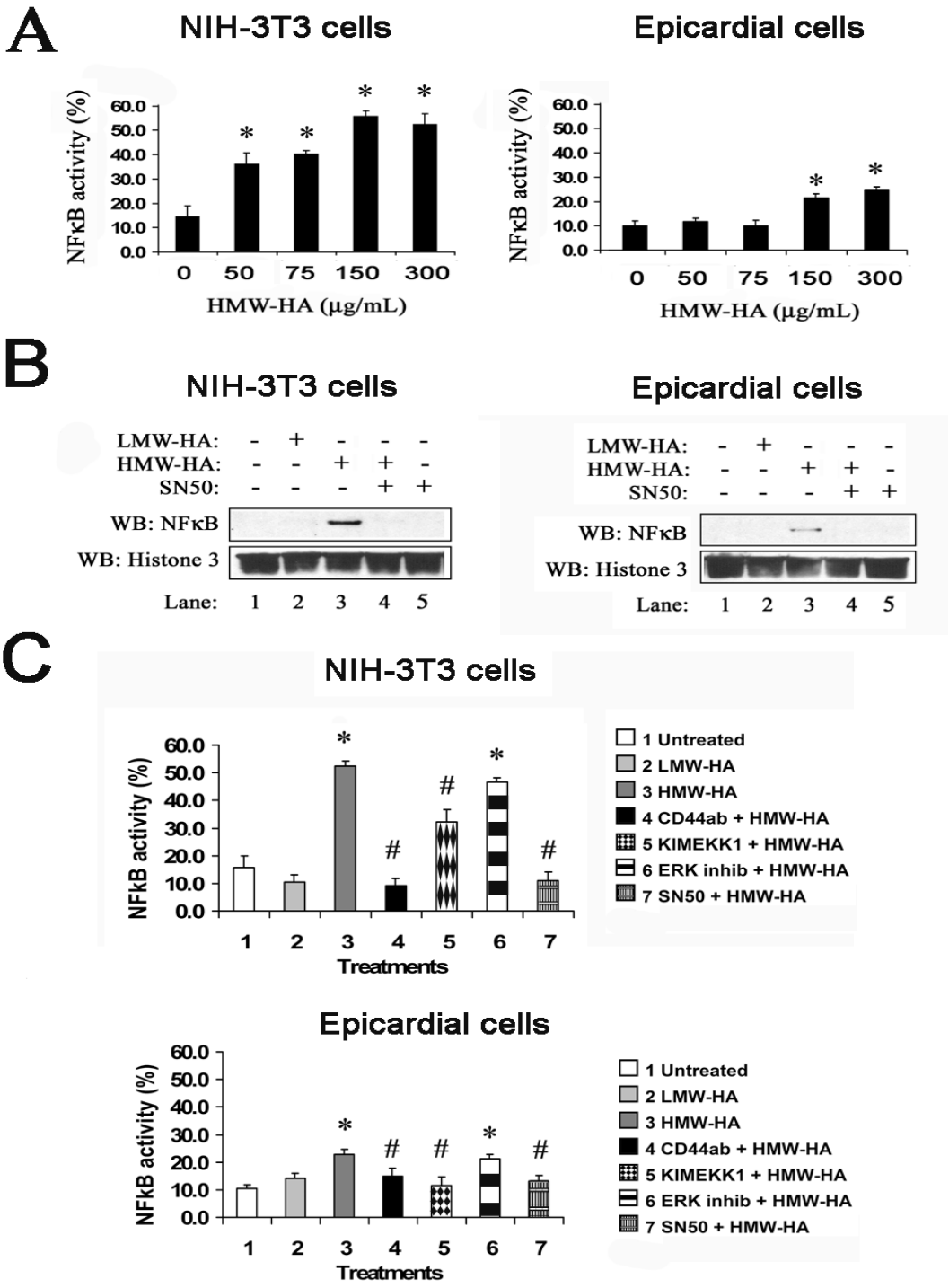

Figure 3.5. CD44 and MEKK1 are required for HMW-HA to induce NFκB. (A) NFκB-SEAP reporter activity was measured in cells following 30 minute stimulation with HMW-HA and 24 hour incubation. (B) Cells left untreated or treated with LMW-HA, HMW-HA alone, HMW-HA in the presence of SN50, or SN50 alone. Nuclear extracts were employed for WB with anti-NFκB and anti-histone 3 antibodies. (C)

NFκB-SEAP reporter activity was measured in cells treated with LMW-HA or HMW-HA in the absence or presence of CD44 blocking antibody, KI-MEKK1, ERK inhibitor peptide or SN50.

Because overexpression of NFκB during development has been shown to induce the expression of several members of the Snail family of transcription factors (173), we explored the potential involvement of Snail2 in HA-mediated responses. For this, we performed RT-PCR and Western blotting following stimulation with HA to detect Snail2 mRNA and protein levels, respectively. Here we show that, in both epicardial and NIH-3T3 cells, HMW-HA induces the expression of Snail2 at the mRNA (Fig. 3.6A) and protein levels (Fig. 3.6B) in a dose-dependent manner. However, similarly to the observed patterns of NFκB activation, the increased Snail2 expression in response to HA was more robust in NIH-3T3 cells.

The importance of HA molecular size for the induction of intracellular signals was also demonstrated as Snail2 mRNA levels are unaffected by LMW-HA treatments (lane 2, Fig. 3.6C). Also, incubation with a CD44 blocking antibody, KI-MEKK1 or SN50, but not the ERK inhibitor, effectively inhibits the induction of Snail2 by HMW-HA (lanes 4-7, Fig. 3.6C). Together, these data indicate that a NFκB/Snail2 pathway is activated in response to HMW-HA and that these signals are transduced via CD44 and MEKK1 but not ERK1/2.

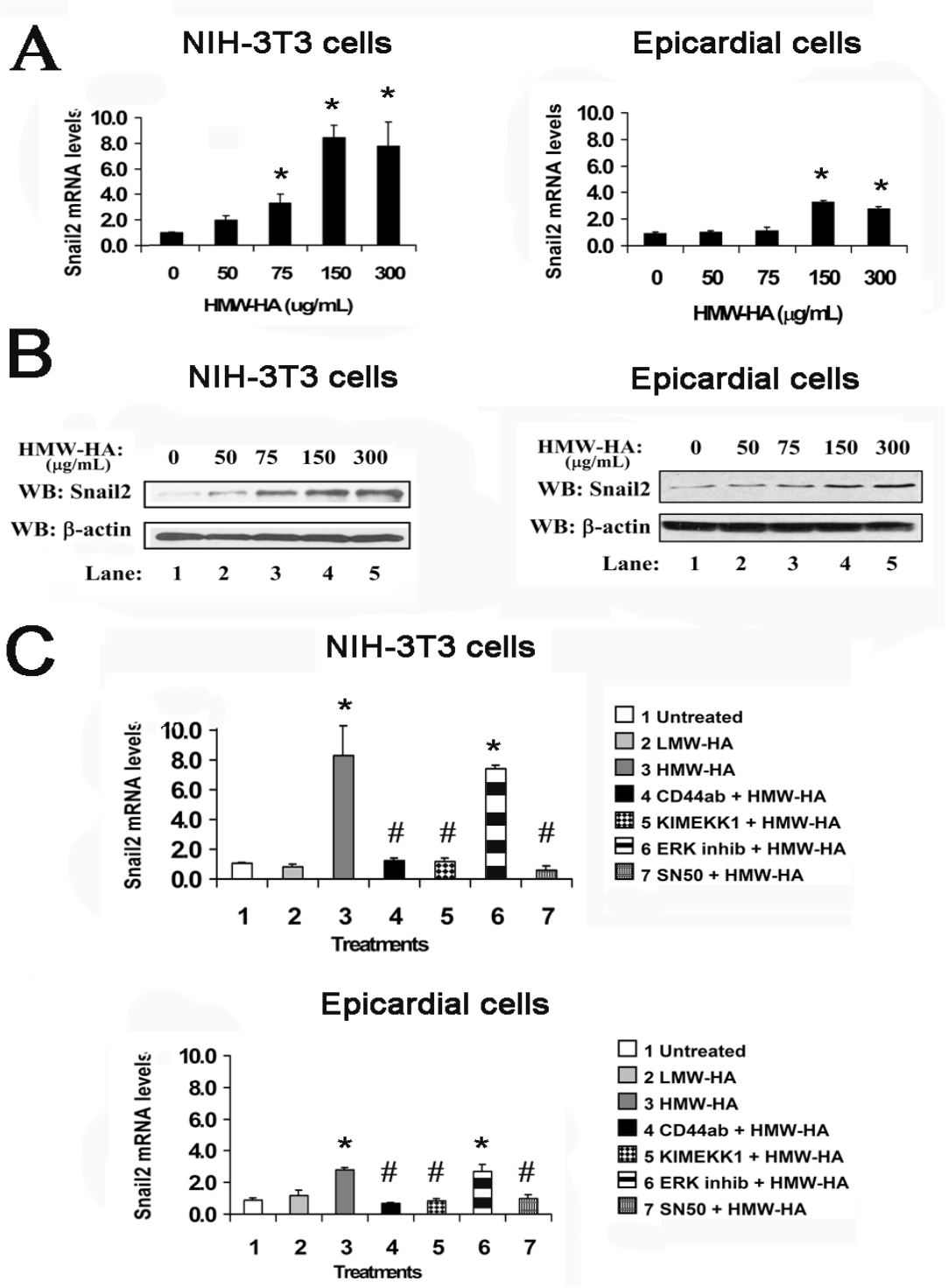

Figure 3.6. CD44 and MEKK1 are required for HMW-HA to induce Snail2 expression. (A) Snail2 mRNA was detected by real-time PCR following stimulation with HMW-HA for 30 minutes and a 24-hour incubation period. (B) Snail2 protein levels were analyzed by WB following stimulation with HMW-HA for 30 minutes and a 24-hour incubation period. (C) Snail2 expression in cells treated with LMW-HA or

HMW-HA in the absence or presence of CD44 blocking antibody, KI-MEKK1, ERK inhibitor peptide or SN50. *, $p<0.05$ as compared to the untreated control; #, $p<0.05$ as compared to treatment with HMW-HA alone.

3.2.4. CD44 and MEKK1 are important for the induction of cellular invasion and differentiation by HA:

We have shown in Chapter 2 that HMW-HA induces embryonic cell differentiation and invasion, both of which are important steps of the EMT process. In this Chapter, we have demonstrated that both CD44 and MEKK1 play a role in the regulation of intracellular signals by HA. Thus, we next sought to determine whether these molecules are important for HA to induce the described changes in embryonic cellular behavior. Incubation of cells with a CD44 blocking antibody or expression of a KI-MEKK1 construct followed by treatments with HMW-HA significantly decreases HA-mediated cellular invasion (Fig. 3.7A).

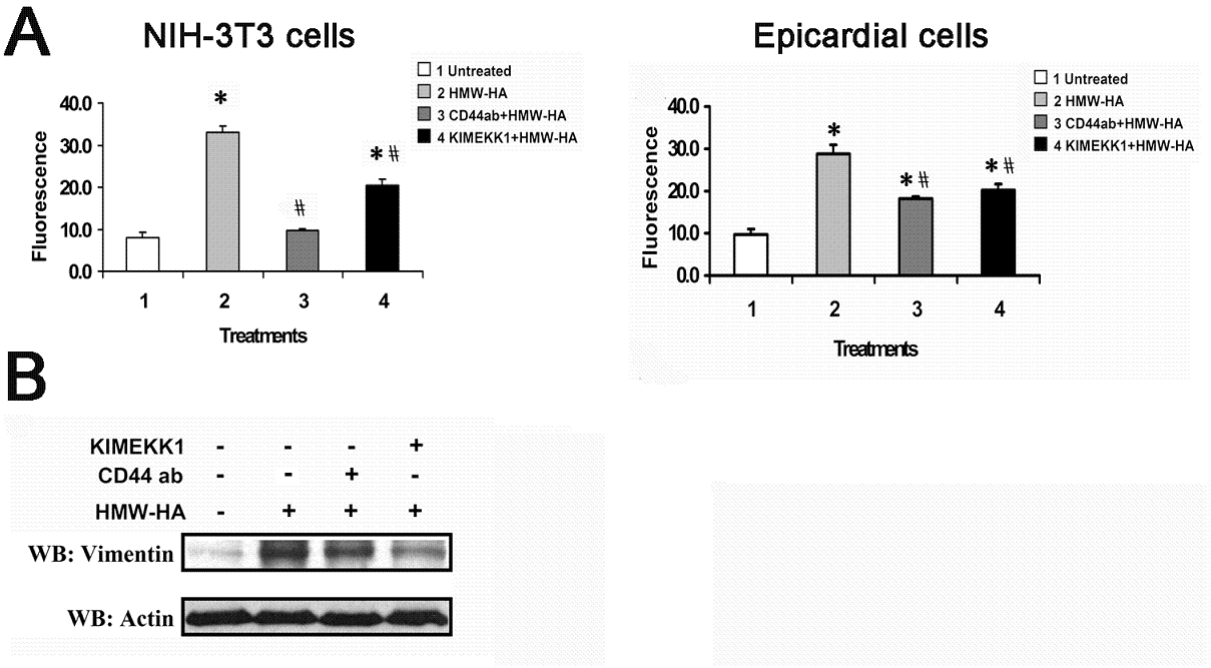

Figure 3.7. The role of CD44 and MEKK1 in HA-mediated cellular invasion and differentiation. (A) Fluorescently labeled cells were stimulated with HMW-HA for 30 min in the presence or absence of CD44 blocking antibody or KI-MEKK1 followed by a 24hr incubation period to allow for invasion. (B) Epicardial cells were treated with HMW-HA for 30 minutes in the presence or absence of CD44 blocking antibody or KIMEKK1 and incubated for 24 hours. Lysates were subjected to WB with anti-vimentin and anti-actin antibodies. *, $p<0.05$ as compared to the untreated control; #, $p<0.05$ as compared to treatment with HMW-HA alone.

Interestingly, the induction of invasion by HA in NIH-3T3 cells appears to occur exclusively through the CD44 receptor, as blockade of this protein completely inhibits the response to HA. In contrast, CD44 appears to play a role in the invasion of epicardial cells but it is likely that other HA receptors may also allow for the transduction of invasive signals in these epithelial cells.

Similarly, blockade of both CD44 and MEKK1 activity in epicardial cells decreases vimentin protein expression (Fig. 3.7B) in response to HMW-HA but does not completely abrogate it. Thus, CD44 and MEKK1 participate in the transduction of HA signals to promote cellular invasion and differentiation but other compensatory mechanisms also participate in this response.

3.2.5. Both the ERK and NFκB pathway mediate HA-induced EMT:

We next studied the contribution of ERK and NFκB effectors during HA-mediated cell motility by treating cells with HMW-HA in the presence or absence of specific inhibitors. As shown in Figure 3.8A, transfection of NIH-3T3 cells with Snail2 siRNA (lane 4), treatment with SN50(lane 5) or ERK inhibitor (lane 6) significantly decreases the response to HA but does not completely abrogate it. On the other hand, in epicardial cells, transfection with control siRNA (lane 3) or Snail2 siRNA (lane 4) or treatment with SN50 (lane 5) does not affect the invasive response to HA. In contrast, incubation with the ERK inhibitor (Fig. 3.8A, lane 6) significantly decreases HA-stimulated invasion. In both cell lines, concomitant addition of the ERK and NFκB inhibitors (lane 7) completely blocks this biological response. Thus, the NFκB pathway appears to be more relevant in NIH-3T3 cells for the induction of HA-mediated cellular invasion.

We also measured the contribution of these pathways to the induction of epicardial cell differentiation by HA through the measurement of changes in vimentin protein levels. Here, only pretreatments with ERK inhibitor alone or a combination of

ERK and NFκB inhibitors were able to abrogate HA-mediated increases in vimentin expression (Figure 3.8B). These observations indicate that the ERK and NFκB cascades work synergistically under the control of HMW-HA to bring about changes in epicardial cell behavior.

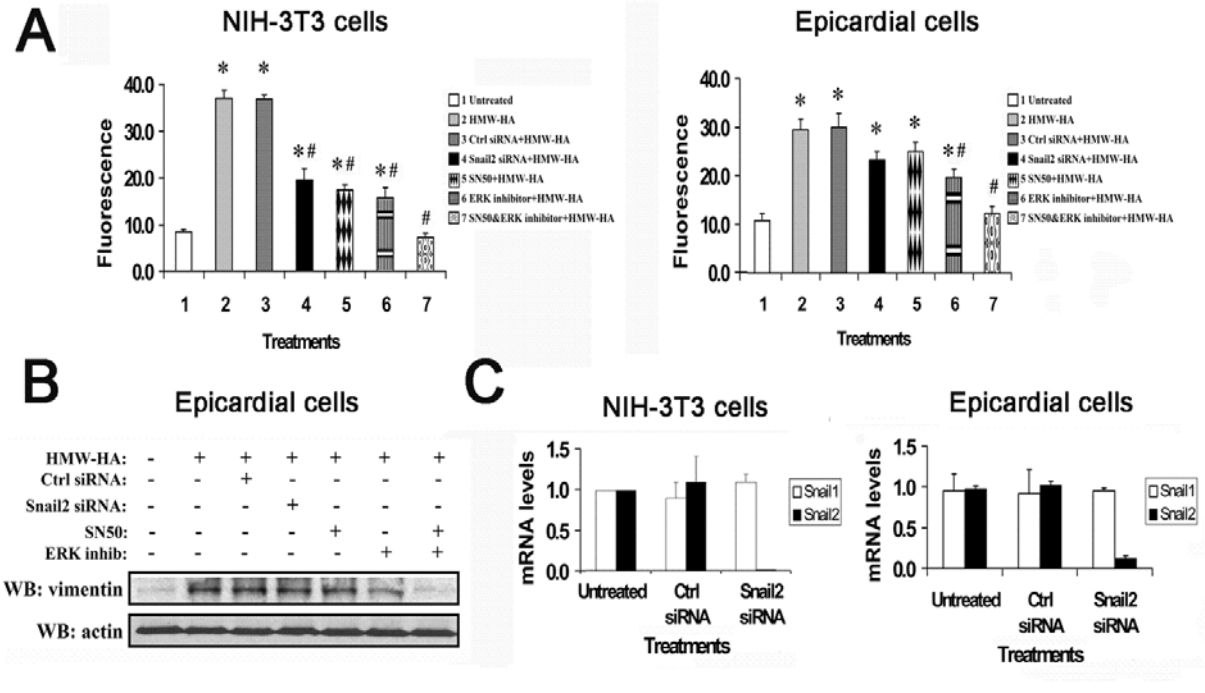

Figure 3.8. Involvement of the ERK1/2 and NFκB cascades in the regulation of cellular invasion and differentiation by HMW-HA. (A) Cells were labeled and treated with HMW-HA for 30 min in the absence or presence of control siRNA, Snail2 siRNA, SN50, ERK inhibitor peptide or SN50 together with ERK inhibitor peptide. After incubation for 24hrs, fluorescence was determined. (B) Cells were subjected to treatments as in 3.8A and lysates were subjected to WB with anti-vimentin and anti-actin antibodies. (C) Real-time PCR was performed to determine specificity and silencing ability of Snail2 siRNA. Both Snail1 (clear bars) and Snail2 (dark bars) expression levels were measured. *, $p<0.05$ as compared to the untreated control; #, $p<0.05$ as compared to treatment with HMW-HA alone.

3.3 Discussion

HA influences a variety of morphogenetic processes and is necessary for proper embryonic development. In addition, HA plays an important role in disease states such as cancer, where elevated levels of this polymer are associated with increased cellular invasion and tumor progression (59, 151). Although several signaling pathways downstream of HA have been identified, the wide range of biological responses affected by this molecule indicates that an even more complex array of signal transduction events is likely to take place.

In trying to determine how HA exerts its biological effects in embryonic cells, we first focused on the role of the HA receptor CD44, as this protein is highly expressed in the heart during embryonic development (167). Binding of HA to CD44 has been associated with the activation of the ERK and NFκB pathways (13, 23), both of which can also be under the control of the MAPK cascade component MEKK1 following several stimuli (128, 161). MAPKs are cytoplasmic serine-threonine kinases that transduce extracellular signals to regulate a variety of physiological responses in a context-specific manner. In the classic arrangement, induction of a receptor leads to activation of a MAPK kinase kinase (MAP3K), which phosphorylates and activates a MAPK kinase (MAP2K), which in turn phosphorylates and activates a MAPK.

Because the MAP3K MEKK1 is also important for epithelial cell motility, we hypothesized that this kinase may participate in the transduction of HA signals to induce embryonic cell invasion. Here we have shown that stimulation with HMW-HA,

but not LMW-HA, induces association of CD44 with MEKK1 and promotes phosphorylation of MEKK1 in NIH-3T3 and epicardial cells (Fig. 3.1). These findings not only demonstrate that MEKK1 is indeed responsive to HA but also highlight a previously unknown interaction between CD44 and MEKK1. Also, these proteins play an important role in HA-mediated EMT as blockade of CD44 or the presence of a kinase-inactive form of MEKK1 significantly impairs the ability of HMW-HA to promote cellular invasion and differentiation (Fig. 3.7). Together, these observations further imply that MEKK1 functions to convey HA signals and thus may lead to the activation of MAPK cascades.

Through our study of possible downstream effectors, we have demonstrated that HMW-HA is able to activate ERK1/2 in NIH-3T3 and epicardial cells. Furthermore, blockade of CD44, the lack of MEKK1 kinase activity or inhibition of MEK1/2 significantly decreases ERK1/2 phosphorylation by HMW-HA (Fig. 3.3). These results are consistent with previous findings in other systems suggesting that HA induces ERK1/2 activation through CD44 and also provides additional evidence for the involvement of MEKK1 in HA-mediated signaling. To further investigate whether HA is able to regulate gene transcription through this MAPK pathway, we analyzed the effect of HMW-HA on serum response element (SRE) activation. The SRE is a 29 bp oligonucleotide responsible for the formation of a complex that regulates the transcriptional machinery of target genes following induction by intracellular kinases such as ERK1/2 (153). Similarly to what was observed with ERK1/2, HMW-HA induces SRE activity through a mechanism requiring CD44 and MEKK1 (Fig. 3.4).

Additionally, pretreatment with an ERK inhibitor completely abrogates the ability of HMW-HA to activate SRE, indicating that SRE is specifically regulated by ERK1/2 in response to HA. It is worth nothing that, although inhibition of MEKK1 signals significantly decreases HA-mediated induction of the ERK1/2/SRE cascade, it does not completely abrogate it. Thus, it is possible that HMW-HA may regulate ERK1/2/SRE through additional pathways that are independent of MEKK1. Although this pathway is induced in both NIH-3T3 and epicardial cells in response to HMW-HA, ERK1/2/SRE activation is more robust in epicardial cells, suggesting that HA may differentially regulate intracellular pathways, depending on the phenotypical characteristics of cells.

One of the proposed signaling effectors following the HA/CD44 interaction in cancer cells is the transcriptional regulator nuclear factor kappaB (NFκB) (74). Also, several lines of evidence suggest that Snail family members are activated by NFκB during both embryonic development and tumor metastasis (25, 173). Therefore, we explored the possibility of this pathway also being activated in embryonic cells in response to HA. Interestingly, NFκB activity and Snail2 expression are both induced in response to HMW-HA in a CD44 and MEKK1 dependent manner (Fig. 3.5 and 3.6). However, this pathway appears to be activated to a lesser extent in epicardial cells when compared to NIH-3T3 cells, suggesting a compensatory but not prevalent role for this NFκB/Snail2 cascade in epicardial cells (Fig. 3.9). Furthermore, inhibition of ERK1/2 does not affect the ability of HA to induce NFκB and Snail2, which indicates that the ERK1/2 and NFκB pathways are distinctly activated by HMW-HA and function

independently of each other. As we investigated the role of these proteins in HA-mediated EMT we noted that, while inhibition of both ERK1/2 and NFκB effectively decreased the cellular response to HMW-HA in NIH-3T3 cells, blockade of the NFκB pathway did not significantly affect HA-mediated responses in epicardial cells (Fig. 3.8). Thus, other mechanisms appear to sufficiently compensate for the absence of NFκB signals in epicardial cells. Interestingly, if both ERK1/2 and NFκB are simultaneously blocked, the invasive response to HA is totally inhibited in both cell lines, indicating a synergistic effect between these two pathways.

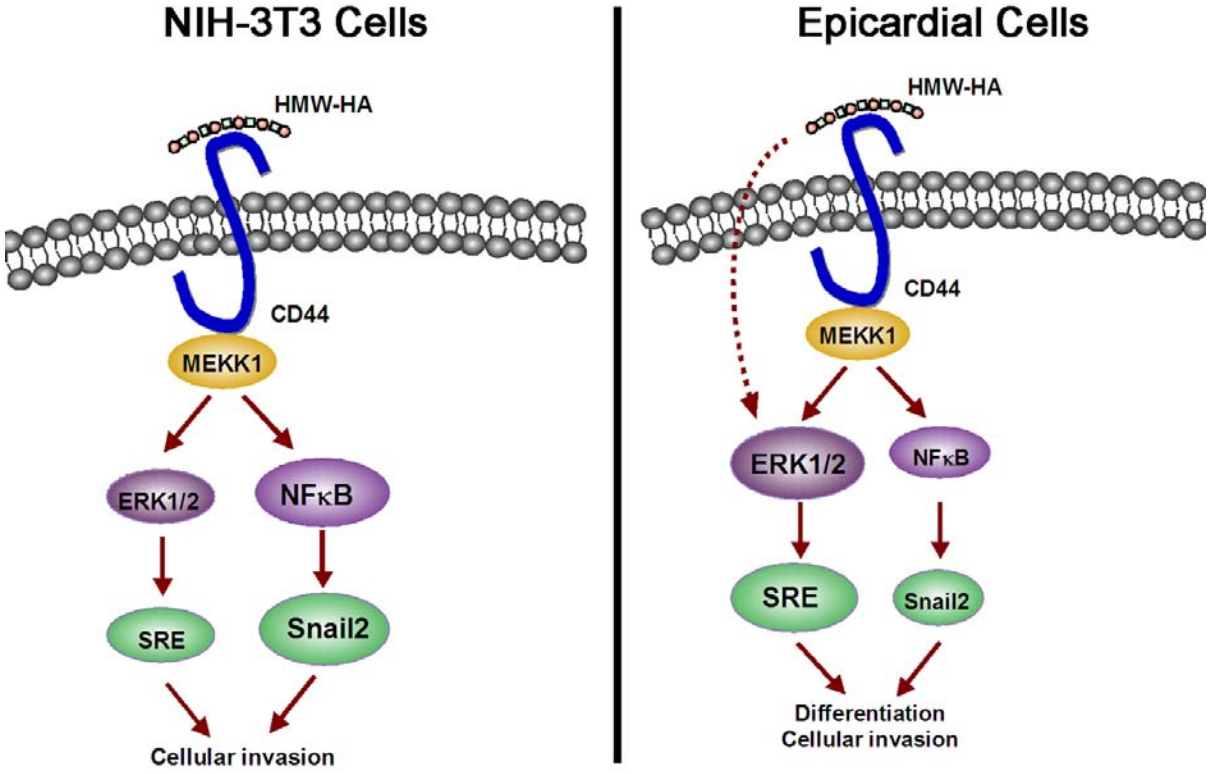

Figure 3.9. Differential regulation of NFκB and ERK pathways by HMW-HA depending on the cell type. In NIH-3T3 cells, HMW-HA induces intracellular signals solely through CD44 and it predominantly activates NFκB-dependent cascades. In epicardial cells, HMW-HA primarily activates ERK1/2 dependent pathways although minor activation of NFκB also occurs. These responses are mediated by CD44 although other cell surface receptors may also collaborate.

This study is the first to our knowledge to demonstrate that HA-mediated activation of NFκB and ERK1/2 is dependent on the CD44/MEKK1 signaling axis and to identify these cascades as crucial for the induction of embryonic cell motility and differentiation (Fig. 3.10). These observations define a novel integrated model for the regulation of embryonic epithelial and mesenchymal cell biology by HA, suggesting a global role for this carbohydrate during development.

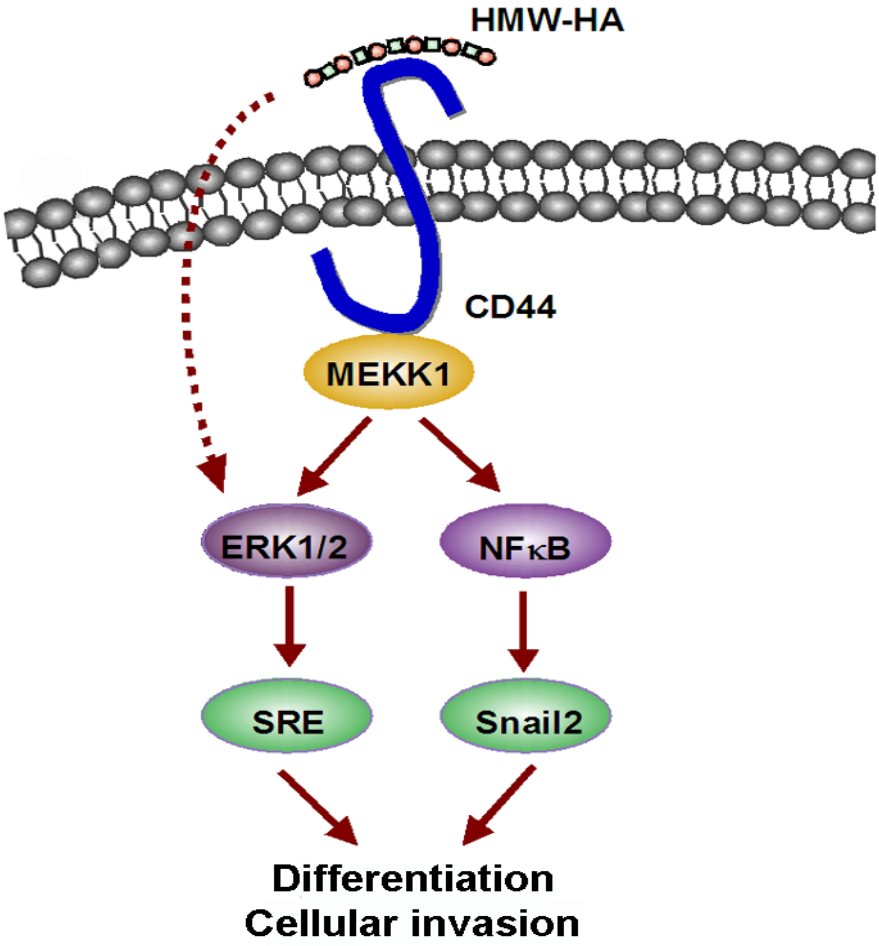

Figure 3.10. A schematic diagram of molecular interactions stimulated by HMW-HA. We propose that HMW-HA activates two distinct signaling cascades, one ERK-dependent and another NFκB-dependent, which lead to the induction of differentiation and invasion in NIH-3T3 and epicardial cells. CD44 and MEKK1 are important for the activation of both cascades, although HMW-HA may also activate ERK1/2 via other mechanisms in epicardial cells.

3.4. Experimental Procedures

Cell culture:

NIH-3T3 mouse fibroblasts and mouse epicardial cells were obtained and cultured as described in Chapter 2. NIH-3T3 cells were maintained at 37°C while epicardial cells were kept at 33°C, which is needed for their conditional immortalization. However, all experiments for both cell lines were performed at 37°C.

Reagents:

High molecular weight hyaluronan (HA) (900- 1,200 kDa; average mass of 980 kDa) and low molecular weight HA (15- 40 kDa; average mass of 31 kDa) were purchased from R&D systems. Rat anti-CD44 blocking antibody (clone KM201), which directly inhibits binding of CD44 to HA (175), was obtained from Southern Biotech (Birmingham, AL) and used at a concentration of 200 ng/mL. The cell permeable NFκB inhibitor SN50 (2 μM) and ERK activation inhibitor peptide (500 nM) were purchased from Calbiochem (San Diego, CA). Antibodies against MEKK1, CD44 and NFκB were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) while those against hemagglutinin and β-actin were from Roche Applied Science (Indianapolis, IN) and Affinity Bio Reagents (Rockford, IL), respectively. Antibodies recognizing histone 3, ERK1/2, Snail2 and vimentin were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against phospho-ERK1/2 and phospho-MEKK1 were obtained from Sigma (St Louis, MO) and Abgent (San Diego, CA).

Expression constructs:

The hemagglutinin-tagged kinase-inactive MEKK1 construct (K1253M) was kindly provided by Dr. Richard Vaillancourt (University of Arizona, Tucson, AZ). Vectors pNFκB-SEAP, pSRE-SEAP, p-CMV-βgal (used for normalization), pTAL-SEAP (SEAP negative control) and pSEAP2 (SEAP positive control) were purchased from Clontech (Mountain View, CA).

SEAP reporter Assays:

NFκB activity was determined by transfecting cells with a pNFκB-SEAP reporter vector expressing a secreted form of placental alkaline phosphatase (SEAP) driven by 4 NFκB consensus sequences in tandem (Clontech). The induction of the serum response element (SRE) was monitored using the pSRE-SEAP construct, which expresses SEAP driven by three tandem copies of the SRE consensus sequence. SEAP activity was normalized for transfection efficiency using a vector coding for β-galactosidase. After treatments and a 24 hr incubation period, SEAP and β-galactosidase expression levels were detected using the Great EscAPe™ SEAP chemiluminescence kit and the luminescent β-gal detection kit, respectively, as indicated by the manufacturer (Clontech).

Immunoprecipitation and Western Blotting:

Cells were serum starved overnight and left untreated or treated with CD44 blocking antibody and/or HA for various time intervals. Subsequently, cells were lysed and diluted in TNEN buffer (1M Tris base, 5M NaCl, 0.5M EDTA and NP40). Samples

were then incubated with anti-CD44 for 1 hr at 4°C and rotated with protein G agarose beads for 2 hrs. Next, beads were centrifugated, washed with TNEN buffer three times and boiled with SDS sample buffer for 5 min.

In some cases, cells were treated with various inhibitors and/or HA, lysed and solubilized in SDS sample buffer. To obtain nuclear extracts, cells were lysed using NE PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL). Total cellular lysates, nuclear lysates, or immunoprecipitates were then resolved by sodium docecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane. After blocking in 3% BSA, membranes were probed with different primary and secondary antibodies. Detection was performed using Super Signal West Pico substrate (Pierce). Quantification by densitometry was performed with the ImageJ program (NIH, Bethesda, MD).

FACE assays:

Cell-based ELISAs were performed using the FACE assay system according to the manufacturer's instructions (Active Motif, Carlsbad, CA). Briefly, cells were seeded into 96-well plates and treated as indicated. Cells were then fixed with formaldehyde and assayed for the presence of total or di-phosphorylated ERK using a colorimetric reaction. Results are expressed as the ratio of di-phosphorylated ERK to total ERK.

Real-time PCR:

After the indicated treatments, Total RNA was isolated from cells using the RNA-STAT60 reagent (Tel-test). cDNA synthesis was conducted using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) according to the manufacturer's instructions. Quantitative real-time PCR was performed on a Lightcycler® 2.0 instrument (Roche Applied Science) using the Lightcycler® Taqman Master Mix (Roche Applied Science). The gene RPS7 was used for normalization. All samples were analyzed in triplicate and a calibrator and negative control were employed for each assay. Cycling conditions included an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. The following primer sequences were employed: *RPS7*: AGCACGTGGTCTTCATTGCT, CTGTCAGGGTACGGCTTCTG; *Snail1*: ACCTGCTCCGGTCTCAGTC, TTGTCAAGGCTGAACCAATG; and *Snail2*, GATCTGTGGCAAGGCTTTCT, ATTGCA GTGAGGGCAAGAGA. All oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). The gene-specific probes were obtained from the Universal Probe Library (Roche Applied Science).

Invasion Assays:

The invasive potential of cells upon HA stimulation was determined by using a transwell chamber system, as described in Chapter 2. Briefly, fluorescently labeled cells were plated in the upper chambers of a transwell 96-well place containing collagen gels. After incubation to allow for adherence, cells were treated with different

inhibitors and/or HA for 30 min, washed with PBS and incubated in low serum media for 24 hours. Invasion was quantified by measuring fluorescently labeled cells that crossed into the lower chamber of the transwell system. Fluorescence was determined at 538 nm with a plate reader (Spectramax Gemini, Molecular Devices, Sunnyvale, CA).

siRNA Experiments:

Small interfering RNA (siRNA) against Snail2 and control siRNA (siRNA-A) were purchased from Santa Cruz Biotechnology. Cells grown to 50% confluence in 6-well plates were transfected with either Snail2 siRNA or control siRNA using XtremeGene siRNA transfection reagent according to the manufacturer's instructions (Roche Molecular Systems). Transfected cells were incubated for 48 hours in medium containing 10% FBS, then harvested by trypsin treatment and used for invasion assays.

Statistical Analysis:

Two sample Student's t tests were performed as appropriate using the Stata 9.0 data analysis and statistical software (StataCorp LP, College Station, TX). The level of significance was established at $P<0.05$.

CHAPTER 4

EGF AND TGFβ2 INDUCE HYALURONAN PRODUCTION AND SUBSEQUENT CELLULAR RESPONSES VIA REGULATION OF HAS2 EXPRESSION AND PHOSPHORYLATION

Portions of this chapter have been adapted from the following publications:

Craig EA, Austin AF, Vaillancourt R, Barnett JV, Camenisch TD. TGFβ2-mediated production of hyaluronan is important for the induction of epicardial cell differentiation and invasion. Exp Cell Research. Epub 2010 Jul 13.

4.1. Introduction

The coronary vascular system is crucial to provide the necessary oxygen, nutrients and tropic signals to support heart tissue development and function. On the other hand, the cardiac valves and septum are important for proper blood flow and partition of the heart into four chambers. Thus, malformation or malfunction of the coronary vessels and valvuloseptal regions can lead to severe ailments such as coronary artery disease and congenital defects, which affect a significant portion of the population (American Heart Association, 2009) (110). During embryogenesis, a subset of cells from the outermost layer of the heart or epicardium generates the different components of the coronary vasculature through a complex process known as epithelial to mesenchymal transformation (EMT) (35). Similarly, endocardial cushion cells undergo EMT to form the precursor tissue that will eventually give rise to

the heart valves and septum (125). The EMT process involves cell detachment from the basement membrane, proliferation, differentiation and invasion through the extracellular matrix (106). Thus, EMT is a crucial mechanism through which progenitor cells become specified and contribute to the formation of the cardiovasculature.

Although many questions remain to be answered in regards to the specific mechanisms that regulate EMT, recent studies have identified transforming growth factor β2 (TGFβ2) and epidermal growth factor (EGF) as important regulators of this process during developmental and disease states (6, 58). TGFβ2 is a member of a large family of structurally related cytokines including activins, bone morphogenic proteins (BMPs) and TGFβs. In mammals, the immediate TGFβ family consists of three isoforms: TGFβ1, TGFβ2 and TGFβ3. However, only TGFβ2 is highly expressed in early stages of epicardial and coronary vascular formation, suggesting a predominant role for TGFβ2 in the regulation of epicardial biology in vivo (94).

TGFβ2 transduces its signals by interacting with heteromeric complexes composed of a type I and a type II TGF-β receptor (TBRI and TBRII, respectively), and in some cases, a type III TGF-β (TBRIII) receptor known as betaglycan (163). However, cellular responses to TGFβ2 are primarily mediated by TBRI and TBRII. Upon binding to TGFβ2, the type II receptor recruits and activates the type I receptor which in turn promotes the activation of downstream signaling molecules, most

notably, the Smad transcription factors. Nevertheless, TGFβ2 signals can also activate members of the MAPK pathway such as p38, JNK and ERK1/2 to induce cellular responses and appear to regulate the expression of hyaluronan synthase 2 (Has2) in highly invasive cells (10, 41, 124). Interestingly, EGF has also been shown to activate MAPK cascades and Has2 to induce EMT and cellular invasion (109, 133), suggesting that various growth factors may work synergistically to regulate the multiple steps of EMT.

Has2 is one of three hyaluronan synthase isoforms found in mammals (Has1, 2 and 3) and is present in the membrane of most mammalian cell types, where it produces the extracellular matrix component hyaluronan (60). Gene knockout studies have shown that Has2 is the most relevant Has isoform during cardiovascular development. While Has1 and Has3 knockout animals are viable and fertile, mice lacking Has2 die at embryonic day (E)9.5 due to severe cardiovascular abnormalities (18). Has2 knockout mice contain virtually no hyaluronan (HA), which prevents the proper formation of the vasculature and endocardial cushions. These alterations in turn cause abnormal blood flow, malformation of the heart valves and septae, and result in premature death.

Because of the early lethality of the Has2 knockout model, it is not known whether Has2 and its product HA are important for developmental events that occur beyond E9.5, such as epicardial and coronary vascular formation. However, evidence from normal embryos and in vitro studies suggests that Has2 and HA may also play a

role in later stages of cardiovascular development. For example, just as in the endocardial cushions, HA is highly abundant in the epicardium, around the time when cells start to undergo EMT (68). Furthermore, HA induces epicardial cell differentiation and invasion in vitro (22).

Despite the relevance of TGFβ2, EGF and Has2 in the regulation of multiple EMT steps, it remains unclear whether signals from these molecules interact and become integrated in a developmental context. Thus, the aim of the present study was to determine whether TGFβ2 and EGF contribute to the regulation of Has2 and define the potential role of these interactions in the induction of biological responses in embryonic cells.

This chapter addresses our third specific aim, which was to define the intracellular effectors that regulate Has2 activation and subsequent hyaluronan production in response to growth factors. Here our goal was to: a) Investigate whether EGF and TGFβ2 are important regulators of hyaluronan synthesis by Has2. b) Identify the mechanisms through which EGF and TGFβ2 participate in the regulation of Has2. c) Determine whether EGF, TGFβ2, and HA-mediated pathways collaborate or work separately to regulate EMT.

We specifically chose to investigate the role of TGFβ2 and EGF in these responses because previous studies have identified these cytokines as crucial for both the regulation of Has2 and the EMT process during normal and pathological

conditions. However, the intracellular mechanisms through which TGFβ2 and EGF may affect Has2 activity and the importance of these interactions has not been defined.

For all experiments in this chapter, TGFβ2 was employed at a concentration of 2 ng/mL and EGF at 20 ng/mL as these concentrations are considered physiologically relevant (33, 58, 64, 91). As in previous chapters, we utilized epicardial and NIH-3T3 cells in all of our studies as a means to compare and contrast the effect of growth factors in cells that are phenotypically different. For the detection of hyaluronan production, we used an ELISA-like method in which the hyaluronan binding protein Aggrecan acts as a capture and detection reagent. This assay allows for the collection of samples at multiple time points in a single experiment and does not require the use of radioactivity, as was the case with older techniques used for hyaluronan quantification. Furthermore, we employed a variety of pharmacological inhibitors, knock down technology, antibodies and enzymes to identify key players for the transduction of EGF and TGFβ2 signals in embryonic cells.

4.2. Results

4.2.1. MEKK3 is activated by TGFβ2 and in turn induces MAPK phosphorylation:

As both TGFβ2 and the MAP3K MEKK3 are necessary for proper cardiovascular development (129, 170), and TGFβ2 has been shown to regulate several members of the MAPK cascade (92), we investigated whether TGFβ2 activates MEKK3 in embryonic cells. A crucial step for MEKK3 activation is the specific phophorylation of

the serine residue at position 526 of this protein (174). Thus we explored whether MEKK3 is phosphorylated at S526 in response to TGFβ2 stimulation. As shown in figure 4.1, treatment with 2ng/mL of TGFβ2 significantly induces MEKK3 phosphorylation at S526 in a time-dependent manner.

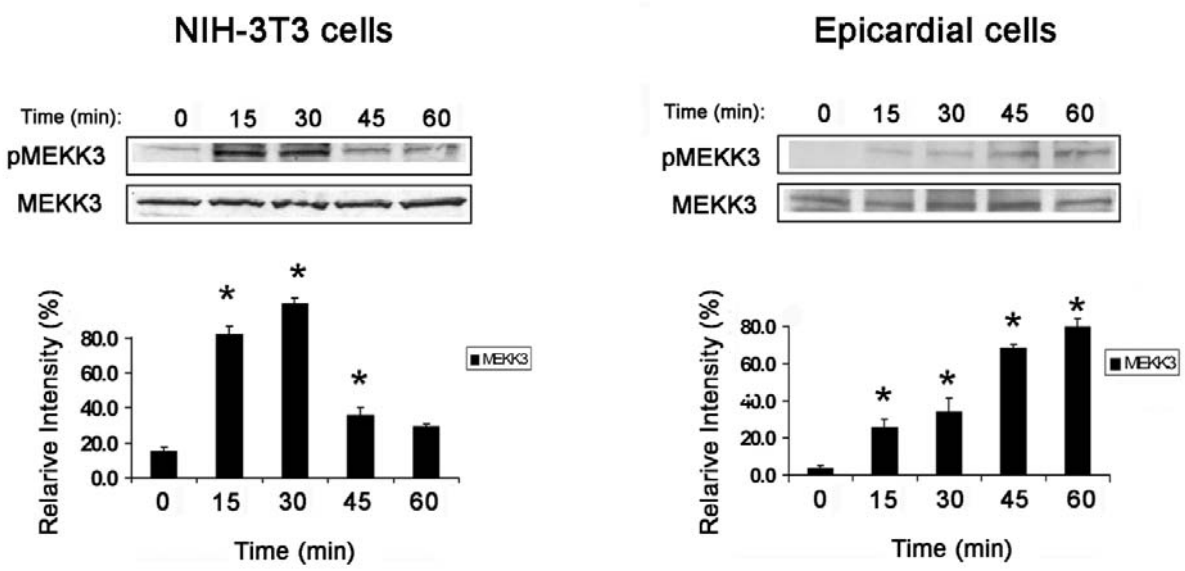

Figure 4.1. Effect of TGFβ2 on MEKK3 phosphorylation. Cells treated with TGF-β2 for 0-60 min were employed for WB with anti-phospho-MEKK3 (top panel) and reblotted with anti-MEKK3 (bottom panel). Densitometry measurements are expressed as phospho-MEKK3 band intensity relative to the MEKK3 bands.

As part of the MAPK relay cascade, activation of MEKK3 has been shown to induce multiple downstream kinases including p38, ERK1/2, SPK/JNK and ERK5 (29, 42, 157). Thus, we investigated whether any of these molecules are also phosphorylated, and thefore activated, in response to TGFβ2. We detect increased phosphorylation of ERK1/2 and p38, but not ERK5 or SPK/JNK, after stimulation of

NIH-3T3 cells with TGFβ2 (Fig. 4.2, left panel). In contrast, ERK1/2 and ERK5, but not p38 or SPK/JNK, are phosphorylated following TGFβ2 stimulation of epicardial cells (Fig.4.2, right panel). This is indicates that TGFβ2 differentially regulates MAPK cascades, depending on the cell type.

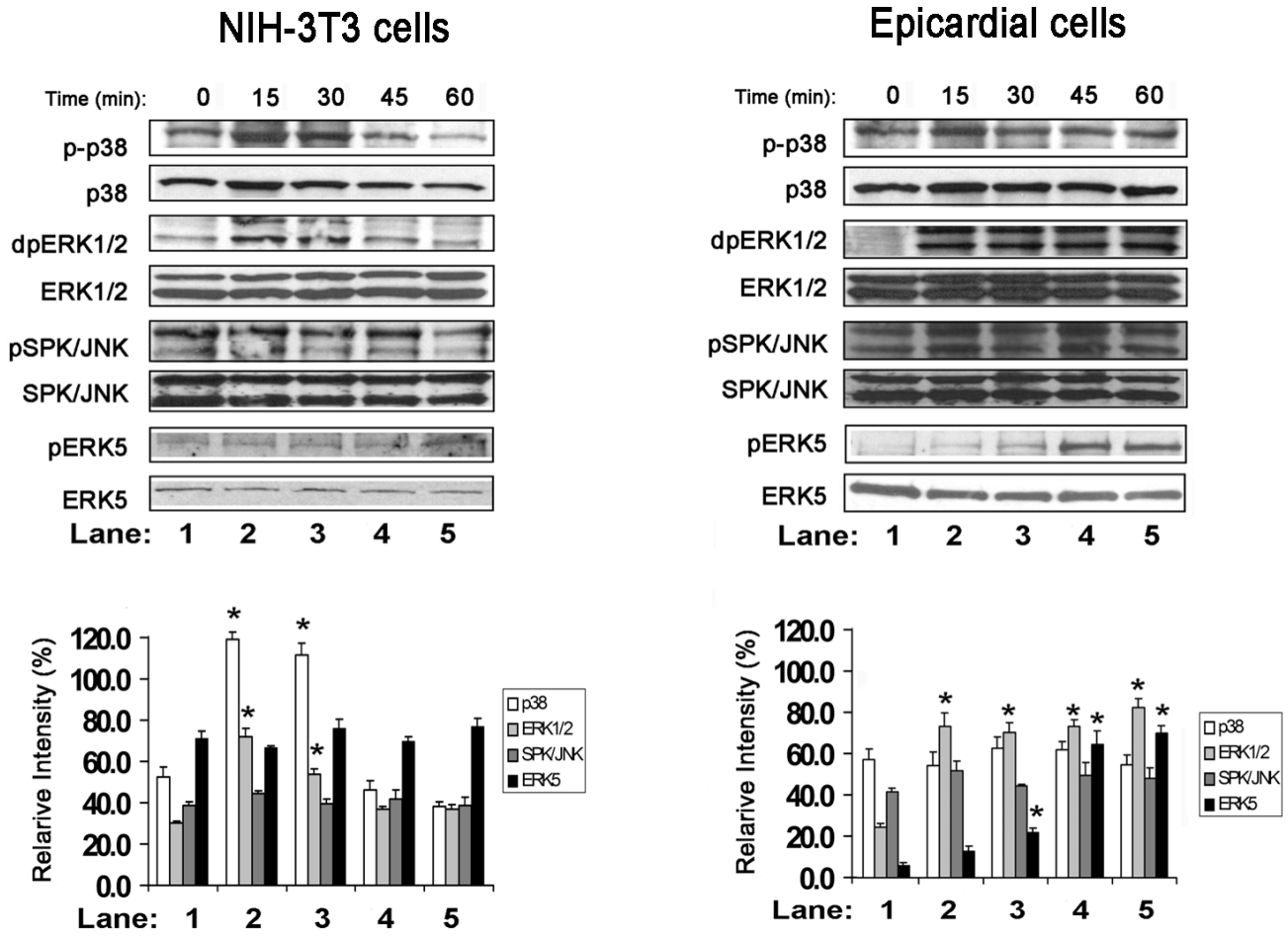

Figure 4.2. MAPK activation in response to TGFβ2. Cells were treated with TGFβ2 for 0-60 min and WBs were performed with antibodies against the phosphorylated and total forms of p38, ERK1/2, SPK/JNK and ERK5. Results were quantified by densitometry and the graphs are displayed as phosphorylated band intensity relative to the total protein bands.

To determine whether MEKK3 is upstream of ERK1/2, ERK5 or p38 following TGFβ2 stimulation, we transfected our cells with kinase inactive (KI) MEKK3 prior to the addition of TGFβ2. KIMEKK3 can not be phosphorylated and therefore can not activate downstream kinases. Thus, if MEKK3 is important for the phosphorylation of ERK1/2, ERK5 or p38, then inactivation of MEKK3 should prevent TGFβ2 from inducing these MAPKs.

As shown in the left panels of figure 4.3A, KI-MEKK3 completely abrogates the ability of TGFβ2 to induce ERK1/2 and p38 phosphorylation in NIH-3T3 cells (lanes 3-6). Similarly, expression of KIMEKK3 significantly inhibited the ability of TGFβ2 to induce ERK1/2 and ERK5 phosphorylation in epicardial cells (Fig. 4.3A, right panels). These observations suggest that the activation of MAPKs in response to TGFβ2 is directly under the control of MEKK3.

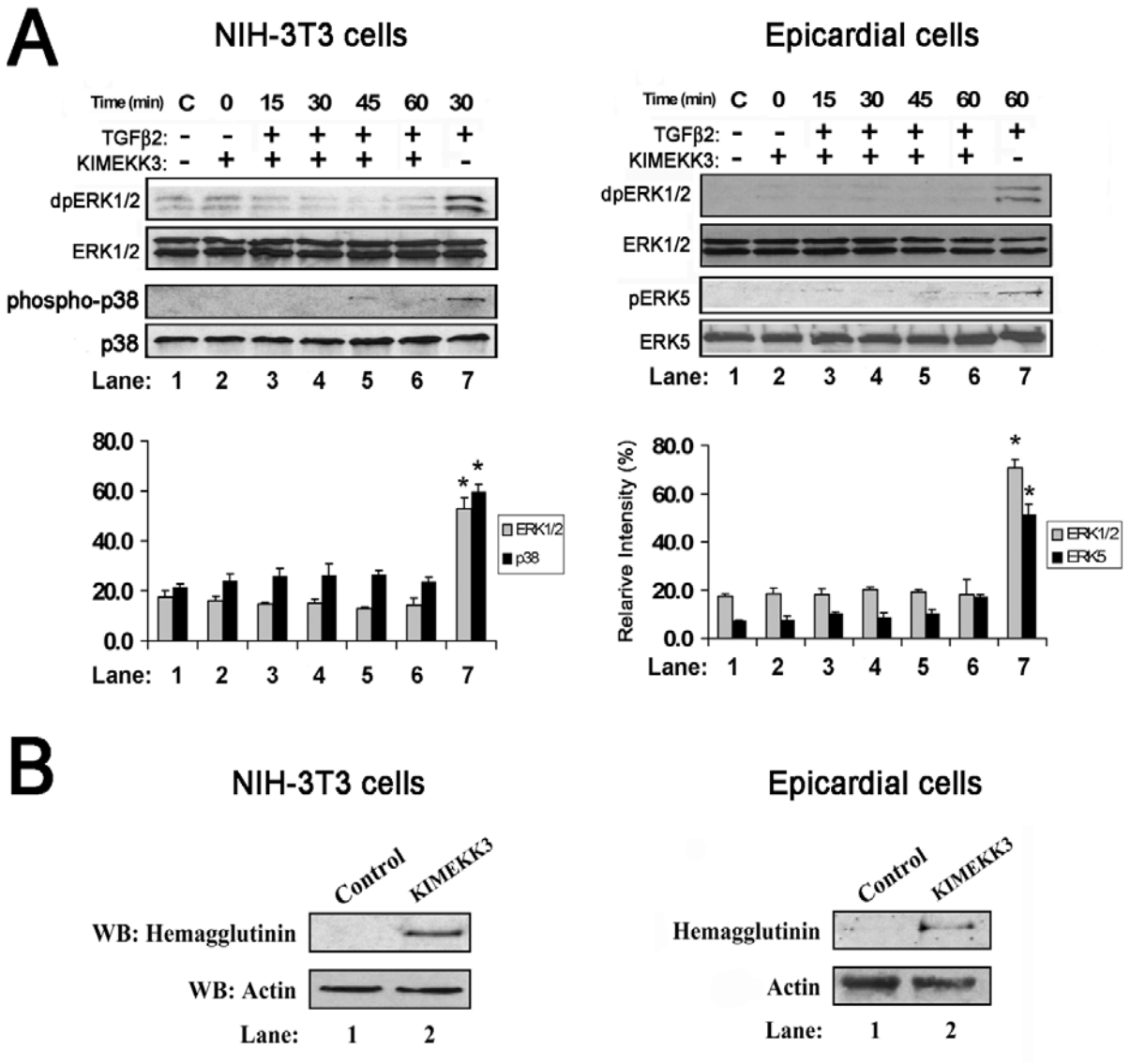

Figure 4.3. MEKK3 mediates TGFβ2-induced activation of MAPKs. (A) Cells were transfected with KI-MEKK3 and then stimulated with TGFβ2 for 0-60 min. WBs were performed with antibodies against the phosphorylated and total forms of ERK1/2, ERK5 and p38. Results were quantified by densitometry and the graphs are displayed as phosphorylated band intensity relative to the total protein bands. *, $p<0.05$ as compared to the untreated controls. (B) Cells were left untransfected or transfected with KI-MEKK3 and incubated for 48 hrs to verify the expression of the hemagglutinin-tagged KI-MEKK3 construct. Lysates were subjected to Western Blotting (WB) with anti-hemagglutinin and anti-actin antibodies.

4.2.2. TGFβ2 promotes Has2 expression through a mechanism requiring MEKK3:

Because Has2 has been shown to be under the control of TGFβ2 in multiple cell types, we sought to determine whether TGFβ2 also regulates Has2 expression in embryonic cells. Here, we performed RT-PCR and Western Blotting to determine the effect of TGFβ2 on Has2 mRNA and protein levels. As shown in figure 4.4A (white bars), TGFβ2 significantly increases Has2 mRNA, with up to a 9 fold increase in NIH-3T3 cells and a 7 fold increase in Has2 message in epicardial cells. These increases appear to be cyclical, suggesting constant turnover of Has2 mRNA.

Furthermore, MEKK3 signals are important in both cell types, as inhibition of MEKK3 kinase activity significantly abrogates TGFβ2-mediated increases in Has2 message (Fig. 4.4A, light grey bars). However, of the MAPKs studied, only ERK1/2 participates in the regulation of Has2 expression in NIH-3T3 cells, as pharmacological inhibiton of ERK1/2, but not p38, decreases the ability of TGFβ2 to increase Has2 mRNA levels (Fig. 4.4.A, top panel, dark grey and patterned bars). On the other hand, ERK1/2 and ERK5 are both important for the induction of Has2 mRNA by TGFβ2 in epicardial cells (Fig. 4.4.A, lower panel, dark grey and patterned bars). These results are coincident with the patterns of MAPK activation by TGFβ2 described in figure 4.2. Also, these observations suggest that MEKK3 may regulate Has2 mRNA levels through additional mechanisms other than MAPK induction.

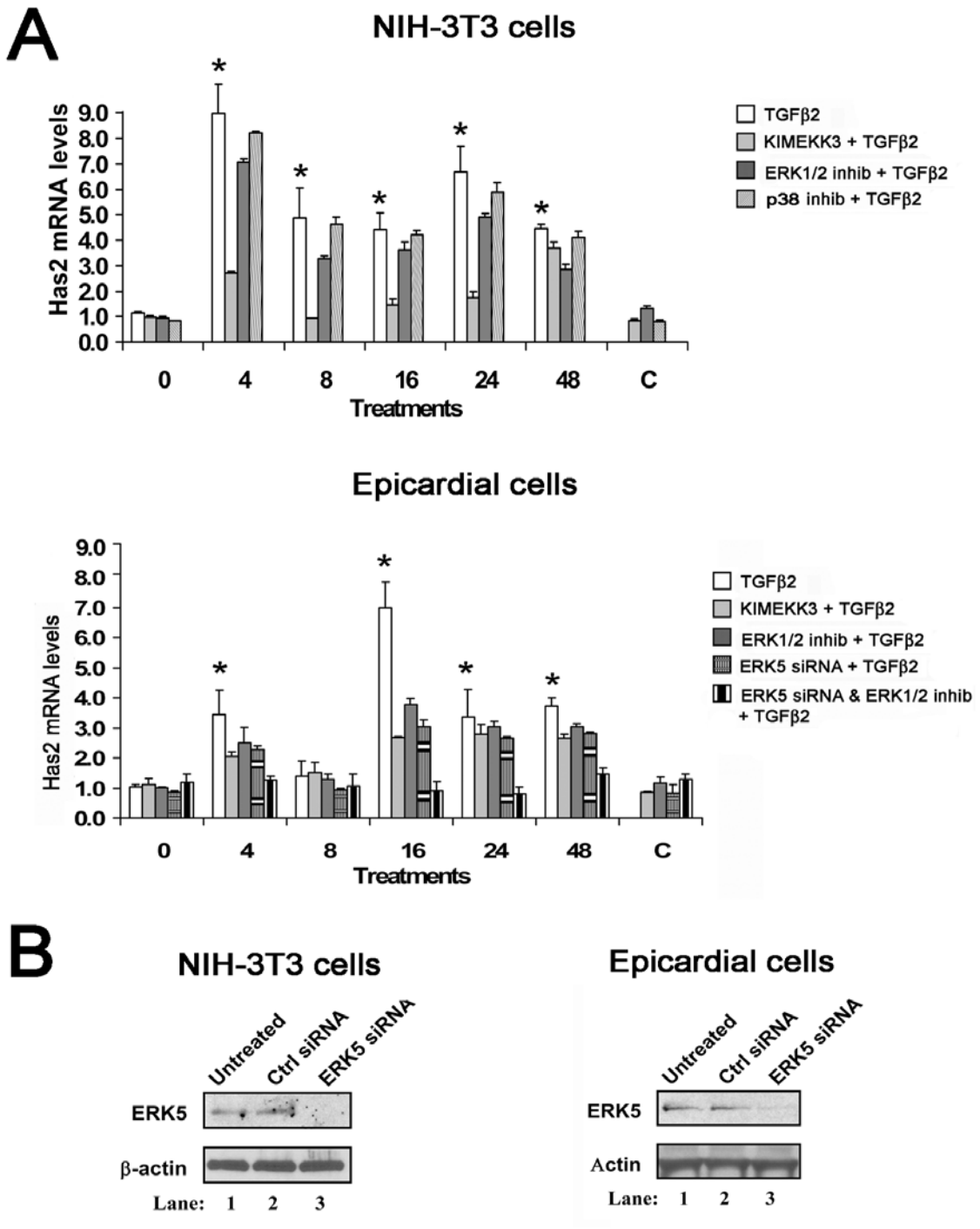

Figure 4.4. Involvement of MEKK3-dependent pathways in the regulation of Has2 message by TGFβ2. (A) Has2 mRNA levels were detected in cells treated with TGFβ2 for 4-48 hrs in the presence or absence of KI-MEKK3, ERK1/2 inhibitor peptide, ERK5 siRNA alone, ERK5 siRNA together with ERK1/2 inhibitor peptide or p38 inhibitor. *, $p<0.05$ as compared to the untreated control. (B) WBs were performed to determine the effect of ERK5 siRNA in silencing ERK5 protein.

Similarly to what we observed with Has2 mRNA, Has2 protein levels are induced in response to TGFβ2 in both cell types in a time-dependent manner (Fig 4.5A). However, this increase in Has2 protein occurs much more rapidly in NIH-3T3 cells, which show significant increases in Has2 as early as 8 hrs post treatment with TGFβ2. These results are coincident with the more robust stimulation of Has2 message observed in NIH-3T3 cells in comparison to epicardial cells.

To assess whether MEKK3-dependent signals are required for the induction of Has2 protein by TGFβ2, we again employed a variety of pharmacological inhibitors and silencing techniques. Transfection with KI-MEKK3 (lane2) or treatment with an ERK1/2 inhibitor peptide (lane3) prior to stimulation with TGFβ2 disrupts the induction of Has2 protein in NIH3T3 cells (Fig. 4.5B, left panel). However, although we have shown that p38 is activated following TGFβ2 stimulation in NIH-3T3 cells, inhibition of this molecule does not affect the regulation of Has2 expression by TGFβ2 (lane 4, left panel). In epicardial cells (Fig. 4.5B, right panels), the presence of KI-MEKK3 (lane4), inhibition of ERK1/2 (lane 5) or silencing of ERK5 with siRNA (lane 6) significantly abrogates TGFβ2-mediated increases in Has2 protein levels. Thus, MEKK3 mediates TGFβ2 signals and differentially activates MAPKs to promote Has2 expression in a cell-specific manner.

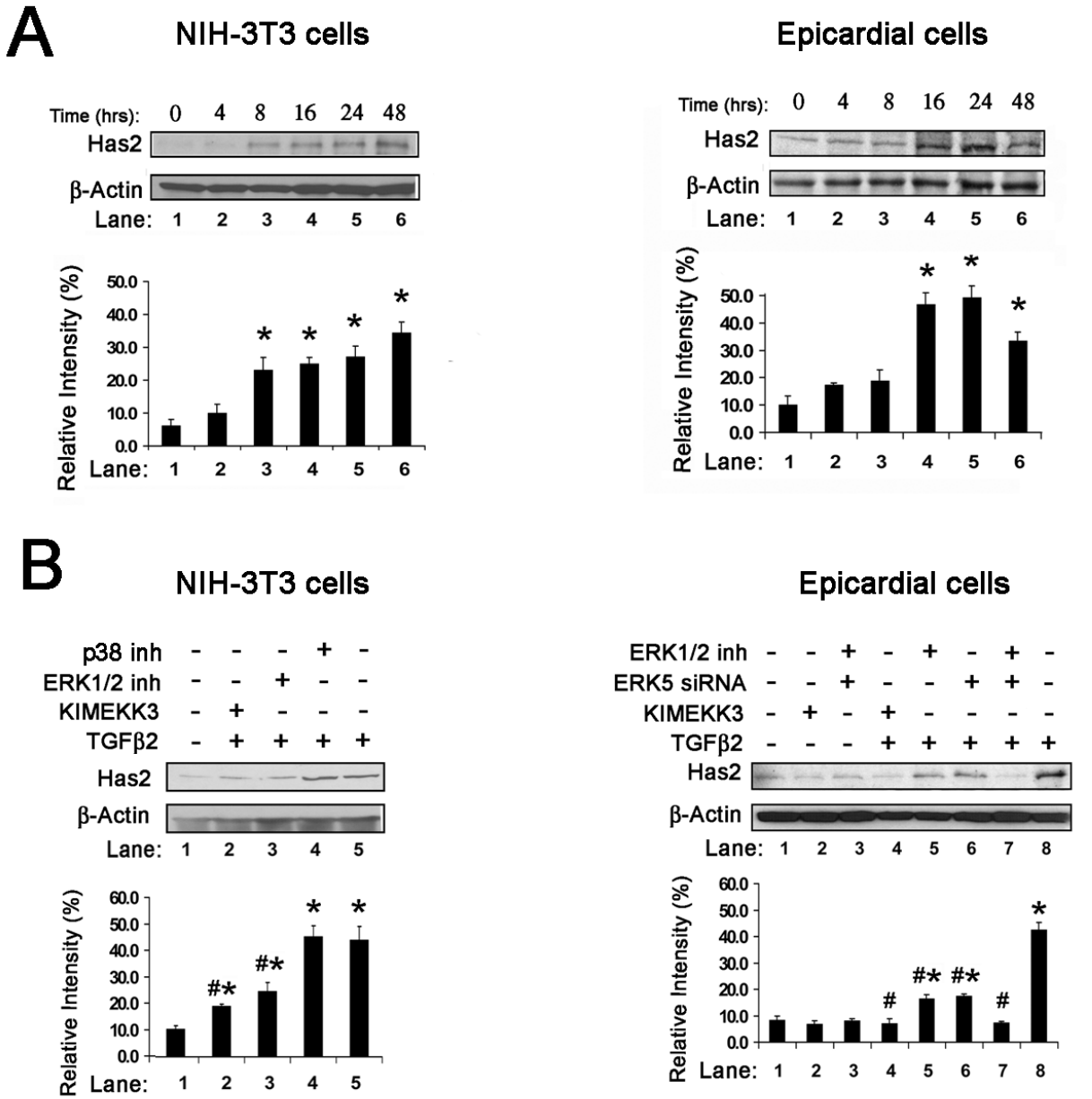

Figure 4.5. Role of MEKK3-dependent cascades in the regulation of Has2 protein levels by TGFβ2. (A) Cells were treated with TGFβ2 (2 ng/mL) for 4-48 hrs and lysates were subjected to WB with anti-Has2 and anti-actin antibodies. The graphs represent WB quantification by densitometry. The results are expressed as Has2 band intensity relative to the actin bands. (B) Cells were transfected with KI-MEKK3 or ERK5 siRNA and/or treated with ERK1/2 inhibitor peptide or p38 inhibitor followed by stimulation with TGFβ2. WBs were performed with anti-Has2 and anti-actin antibodies. *, $p<0.05$ as compared to the untreated control; #, $p<0.05$ as compared to treatment with TGFβ2 alone.

4.2.3. TGFβ2 induces HA production via MEKK3-dependent pathways:

Although TGFβ2 has been shown to induce HA biosynthesis in cancer cells (103), the specific intracellular signals that govern this response or whether this process also occurs in a developmental context remains to be elucidated. Thus, we investigated whether TGFβ2 is able to regulate HA production in embryonic cells. We also explored involvement of MEKK3 and its downstream signals, as we have demonstrated that this molecule is crucial for Has2 expression in response to TGFβ2 stimulation. For this purpose, we quantified the presence of HA in the cell culture media following stimulation with TGFβ2 and/or various inhibitors using an ELISA-like method.

As shown in figure 4.6 (top graph), TGFβ2 induces a significant increase in HA production (white bars) and transfection with a kinase-inactive MEKK3 construct (KI-MEKK3) (light grey bars) or pretreatment with an ERK1/2 inhibitor peptide (dark grey bars) significantly decreases this response in NIH-3T3 cells. TGFβ2 also induces HA production (white bars) in epicardial cells (Fig. 4.6, bottom graph). Inhibition of MEKK3 (light grey bars), ERK1/2 (dark grey bars) or ERK5 (patterned bars) disrupts the ability of TGFβ2 to induce HA synthesis in epicardial cells. These findings suggest that MEKK3-dependent pathways play an important role in the regulation of HA production by TGFβ2.

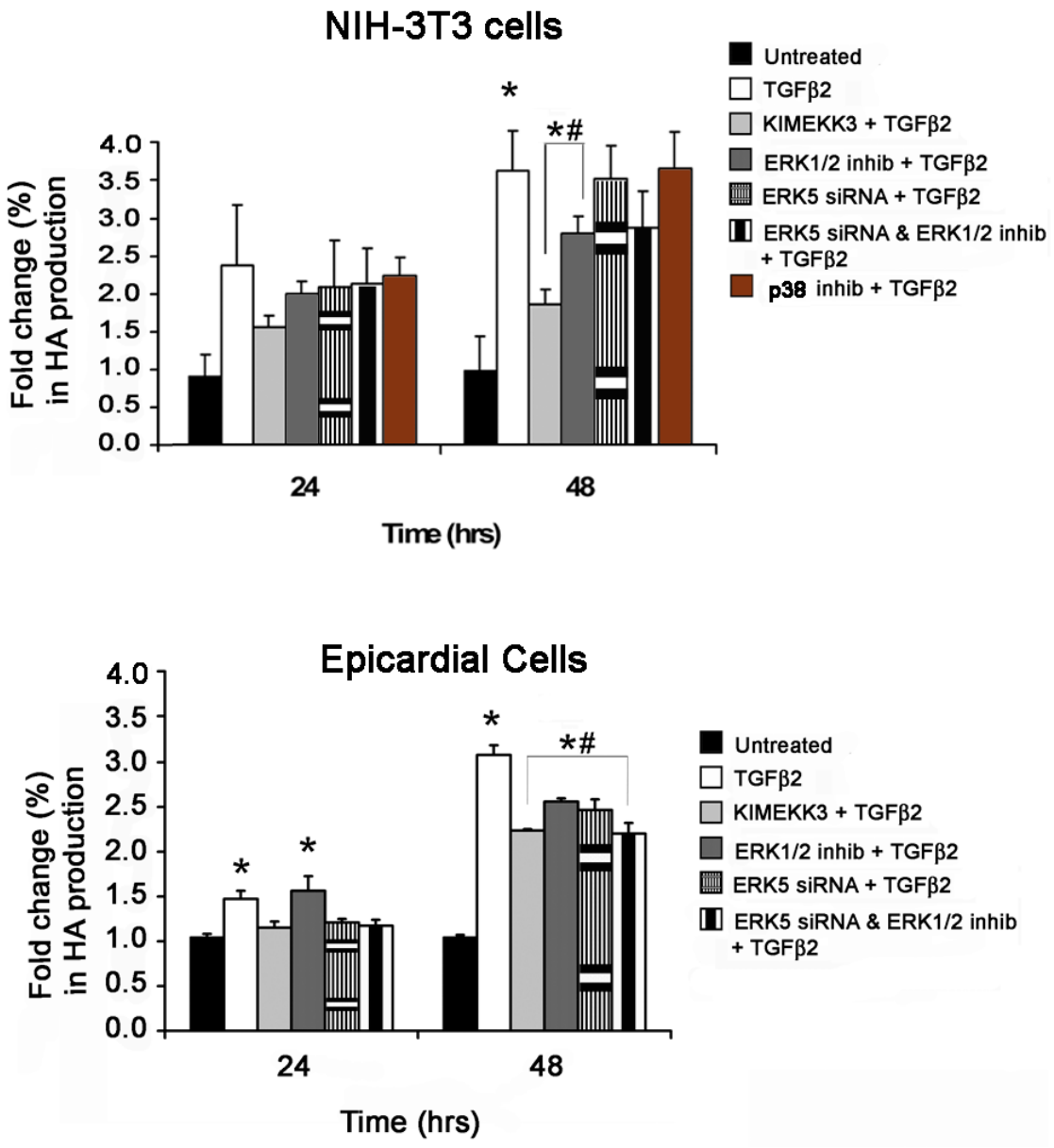

Figure 4.6. Regulation of Hyaluronan production by TGFβ2-mediated signals. Cells were treated with TGFβ2 (2 ng/mL) in the presence or absence of KI-MEKK3, ERK1/2 inhibitor peptide, ERK5 siRNA alone, ERK5 siRNA in combination with ERK1/2 inhibitor peptide or p38 inhibitor. Total amounts of HA secreted into culture supernantants were quantitated after 24 and 48 hrs. The results are shown as fold change in HA production as compared to the untreated controls. *, $p<0.05$ as compared to the untreated control; #, $p<0.05$ as compared to treatment with TGFβ2 alone.

4.2.4. MEKK3 and HA play a role in TGFβ2-mediated cellular differentiation and invasion:

Depending on the cellular and physiological context, TGFβ2 as well as HA-dependent signaling cascades have been shown to induce cell proliferation, differentiation and invasion (6, 22, 95, 118). However, it is not known whether these pathways converge or work independently to induce these cellular responses. Furthermore, as we have shown that TGFβ2 induces the MEKK3 pathway and promotes HA production by Has2, we sought to determine whether these molecules are able to regulate embryonic cell biology.

First we looked at cellular differentiation in our epicardial cells, which are epithelial in phenotype and can act as progenitors for a variety of cardiac cells. As shown in figure 4.7 (lane 2), stimulation of epicardial cells with TGFβ2 leads to an increase in the expression of the mesenchymal cell marker vimentin (red) as early as 24hrs post-treatment. This response is decreased in the presence of KI-MEKK3, ERK1/2 inhibitor peptide or ERK5 siRNA alone. (Fig 4.7, lanes 3, 4 and 5, respectively). Interestingly, knockdown of ERK5 by siRNA together with pharmacological inhibition of ERK1/2 (lane 6), disrupts the response to TGFβ2 considerably more than when only one of these molecules was inhibited, indicating a synergistic effect between ERK1/2 and ERK5 in the transduction of TGFβ2 signals. Additionally, degradation of HA by hyaluronidase (lane 7) and blockade of the HA receptor CD44 (lane 8) also abrogates the ability of TGFβ2 to increase vimentin

expression. Thus, production and size of the HA polymer are important for TGFβ2 to induce morphological changes in epicardial cells.

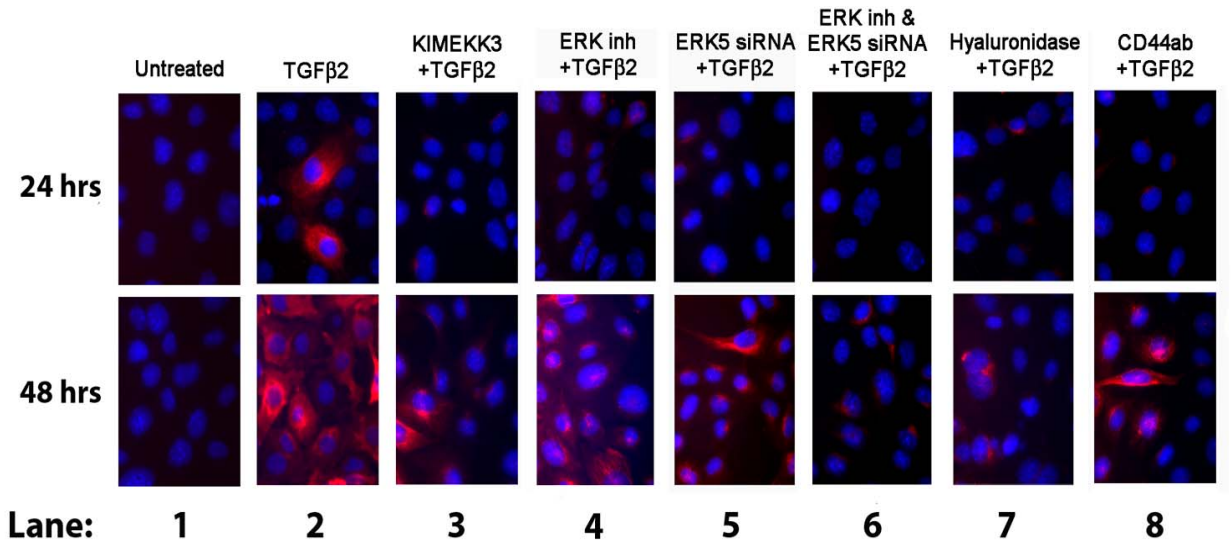

Figure 4.7. Involvement of HA and MEKK3-dependent pathways in TGFβ2-induced differentiation of epicardial cells. Cells were left untreated, treated with TGFβ2 alone or TGFβ2 in the presence of KI-MEKK3, ERK1/2 inhibitor peptide, ERK5 siRNA, CD44 blocking antibody or hyaluronidase. Cells were then fixed and immunofluorescently stained with an antibody against vimentin (red) and bisbenzamide (blue) for nuclear staining.

Next, we tested the role of TGFβ2 in epicardial and NIH-3T3 cell motility by performing a modified Boyden chamber assay. Here we demonstrate that TGFβ2 induces cellular invasion (Fig. 4.8A, white bars) and that pretreatment with KI-MEKK3 or ERK1/2 inhibitor peptide (light and dark grey bars) significantly diminishes this response. However, ERK5 siRNA is only able to disrupt TGFβ2-mediated invasion of epicardial cells and not NIH-3T3 cells (patterned bars), which is coincident with the

selective activation of ERK5 in epicardial cells described in figure 4.2. Similarly, inhibition of p38 does not appear to have an effect in this response (dark blue bars). Lastly, pretreatment with hyaluronidase (light blue bars) or with CD44 blocking antibody (yellow bars) effectively abrogates the ability of TGFβ2 to promote cell motility. These observations support a role for MEKK3 and HA-dependent pathways in the regulation of NIH-3T3 and epicardial cell invasion by TGFβ2.

Next, we assessed whether this enhanced invasion is the result of increased cell number by determining the role of TGFβ2 on cell growth through a MTT assay. As shown in figure 4.8B, stimulation with TGFβ2 for 24 or 48 hrs does not significantly affect NIH-3T3 or epicardial cell proliferation. Thus, TGFβ2 specifically enhances the ability of cells to invade without promoting cell growth.

Together, our data indicate an important functional connection between TGFβ2 and HA-mediated signals and underscores the role of these interactions in the regulation of embryonic cell biology, specifically throughout the EMT process.

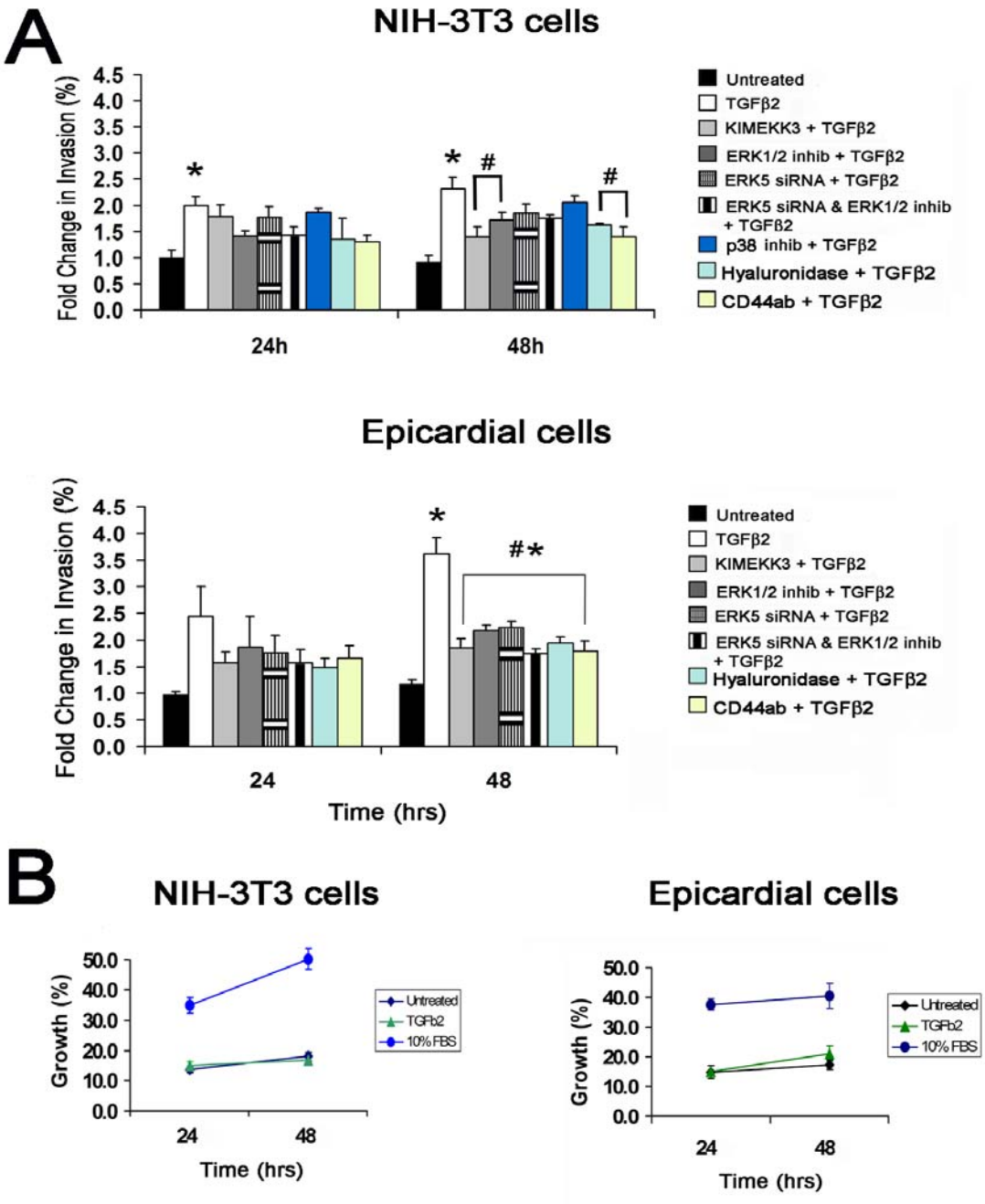

Figure 4.8. Role of HA and MEKK3-dependent cascades in the regulation of embryonic cell invasion by TGFβ2. (A) Fluorescently labeled cells were stimulated with TGFβ2 in the presence or absence of various inhibitors and incubated for 24 or 48 hrs to allow for invasion into collagen gels and through an 8μm pore membrane (B) Cell proliferation was evaluated following stimulation with TGFβ2 for 24 and 48 hrs using the MTT assay. 10% FBS was used as a positive control. *, $p<0.05$ as compared to the untreated control; #, $p<0.05$ as compared to treatment with TGFβ2 alone.

4.2.5. EGF differentially regulates Has2 activity and HA production in NIH-3T3 and epicardial cells:

Similarly to TGFβ2, EGF has been shown to regulate HA synthesis via hyaluronan synthases in multiple cell lines (126). For this reason, we investigated whether EGF also exerts a regulatory effect on Has2 biology in embryonic cells. As shown in figure 4.9A, EGF induces Has2 mRNA levels in both NIH-3T3 and epicardial cells (black bars). However, while the increases in Has2 following EGF stimulation appear to be time-dependent in NIH-3T3 cells, Has2 mRNA peaks at 4 hours in epicardial cells and then rapidly decreases back to basal levels. Thus, EGF differentially regulates Has2 expression, depending on the cell type.

Furthermore, we assessed the role of MEKK3 in the transduction of EGF signals as we have shown that this kinase is necessary for the induction of Has2 by TGFβ2. Nevertheless, blockade of MEKK3 activity with KI-MEKK3 did not significantly affect the ability of EGF to increase Has2 mRNA levels (Fig. 4.9A, grey bars), indicating that MEKK3 does not participate in the regulation of Has2 by EGF. Additionally, we performed Western Blots to determine the effect of EGF on Has2 protein levels. As shown in figure 4.9B, EGF also promotes increases in Has2 protein and these are coincident with the observations at the mRNA level.

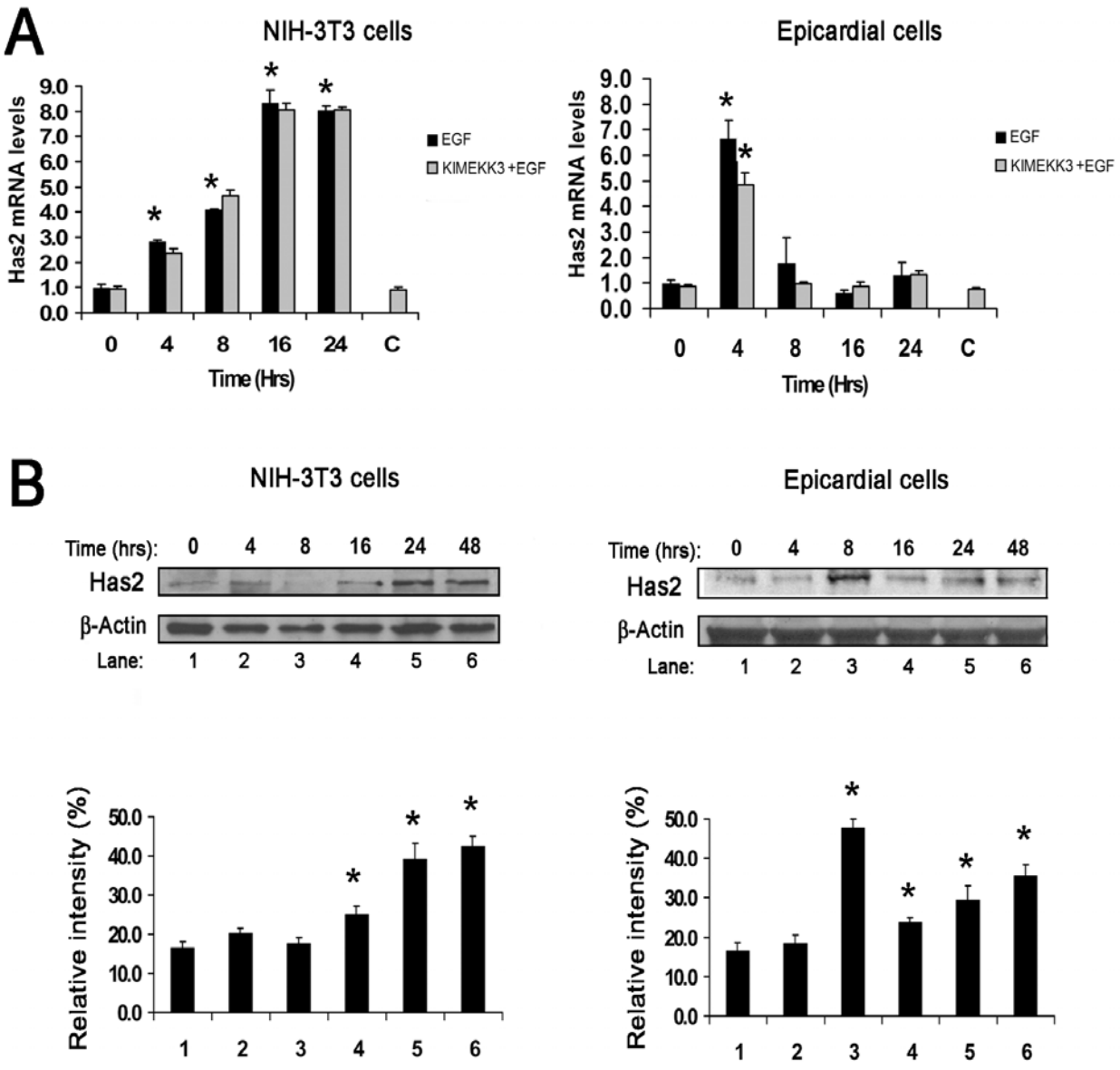

Figure 4.9. Effect of EGF on Has2 expression. (A) Has2 mRNA levels were detected in cells treated with EGF for 4-24 hrs in the presence or absence of KI-MEKK3. (B) Cells were treated with EGF (20 ng/mL) for 4-48 hrs and lysates were subjected to WB with anti-Has2 and anti-actin antibodies. The graphs represent WB quantification by densitometry. The results are expressed as Has2 band intensity relative to the actin bands. *, $p<0.05$ as compared to the untreated control.

Has2 phosphorylation is a crucial step for the activation of the protein's synthetic ability leading to HA production (54, 160). Therefore, we investigated whether EGF modulates this post-translational modification of Has2. As depicted in figure 4.10A, stimulation of both NIH-3T3 and epicardial cells with EGF for 15-60 minutes significantly increases Has2 phosphorylation in tyrosine residues. Thus, EGF not only affects Has2 expression, but also directly regulates its postranslational modification through the addition of phosphate groups.

The involvement of src kinases in EGF-mediated Has2 phosphorylation was also assessed in epicardial cells. These experiments were performed due to initial evidence suggesting that src kinases are able to promote hyaluronan synthase phosphorylation and HA production in cells that have undergone malignant transformation (97, 114). Pretreatment of cells with PP2, a pharmacological inhibitor of src, significantly abrogates the ability of EGF to phosphorylate Has2 (Fig. 4.10B). This inhibition is dose-dependent and suggests a role for src kinases in the regulation of Has2 activity.

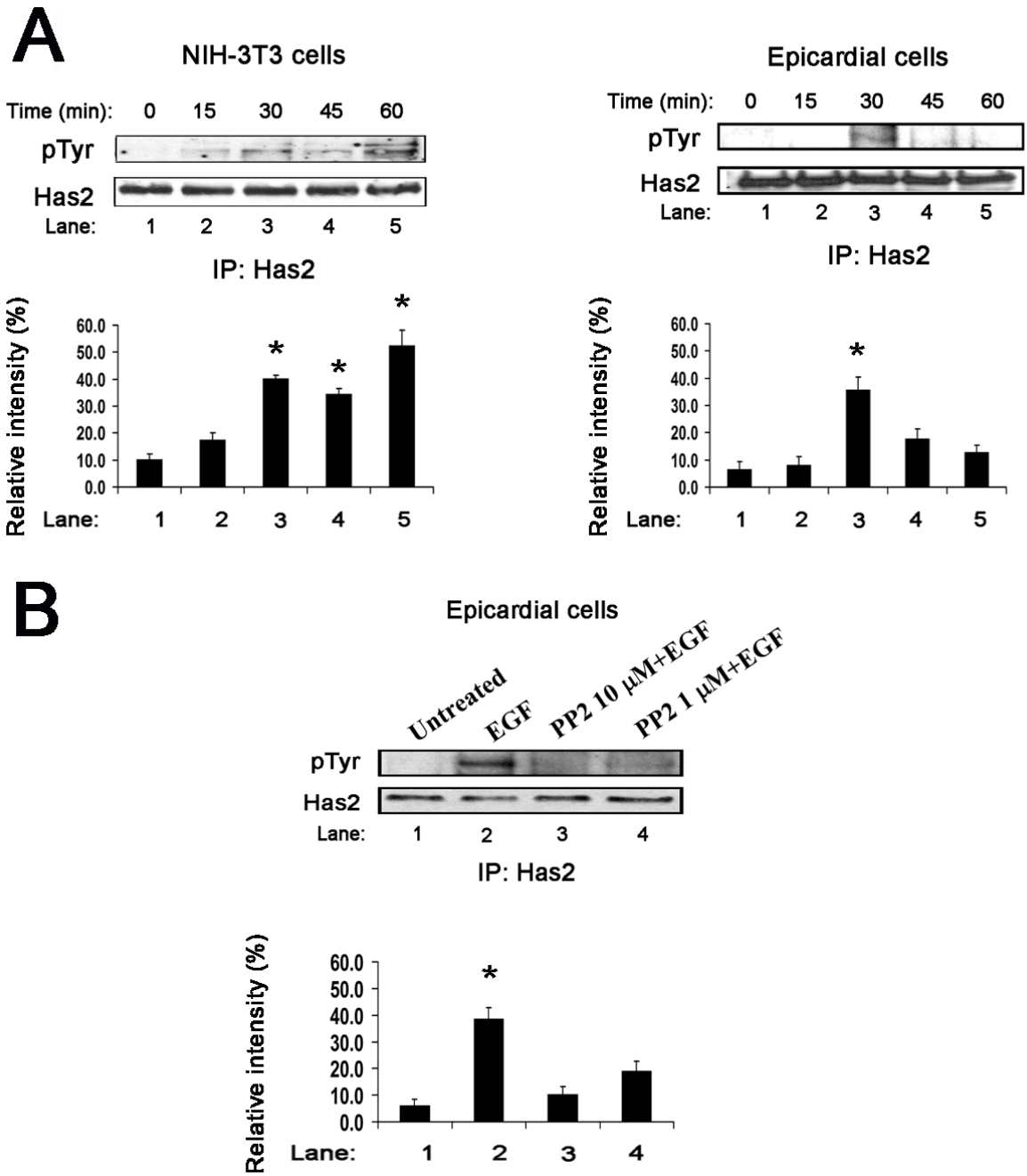

Figure 4.10. EGF promotes Has2 phosphorylation. (A) Cells were treated with EGF (20 ng/mL) for 15-60 min and lysates were subjected to WB with anti-phospho-tyrosine and anti-Has2 antibodies. The graphs represent WB quantification by densitometry. The results are expressed as p-tyr band intensity relative to the Has2 bands. (B) Epicardial cells were treated with PP2 for 1hr previous to stimulation with EGF for 30 min. WB and densitometry analysis were performed as in A. *, $p<0.05$ as compared to the untreated control.

Because Has2 protein expression and phosphorylation is increased in response to EGF, we next investigated whether these changes in Has2 biology result in enhanced HA synthesis. For this, we measured HA concentrations in the cell media with ELISA-like assays and observed that EGF significantly induces HA production in both NIH-3T3 and epicardial cells. However, HA accumulation appears to occur at a faster rate and at higher levels in epicardial cells. These differences may be due to phenotypical variations between the two cells lines or to rapid turnover of HA in the fibroblast environment due to the presence of hyaluronidases. These data indicate that EGF participates in the modulation Has2 activity and subsequent HA production during developmental processes. Furthermore, we have shown that both fibroblastic and epithelial cell lines are able to produce HA endogenously, and thus HA may exert its effects in an autocrine manner.

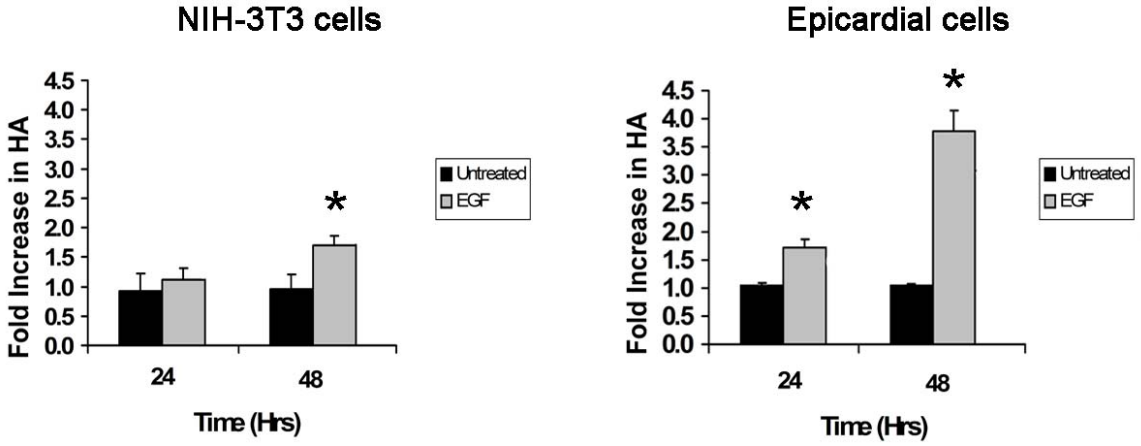

Figure 4.11. Regulation of Hyaluronan production by EGF. Cells were treated with EGF (20 ng/mL) and HA concentrations were measured in culture supernantants collected after 24 and 48 hrs. The results are shown as fold change in HA production as compared to the untreated controls. *, $p<0.05$ as compared to the untreated control.

4.2.6. EGF induces embryonic cell differentiation, invasion and proliferation:

EGF has been shown to regulate multiple steps of EMT in cancer and normal adult cells (49, 159). For this reason, we assessed whether EGF is able to induce biological responses in embryonic cells. We employed the mesenchymal cell marker vimentin as a way to determine cellular differentiation in response to EGF and found that EGF increases vimentin expression in epicardial cells after 24 and 48 hr treatments (Fig. 4.12A). Next, we performed our three-dimensional invasion assays and we showed that EGF significantly induces cellular invasion in both NIH-3T3 and epicardial cells (Fig. 4.12B, black bars). Blockade of MEKK3 kinase activity did not affect the ability of EGF to induce cellular invasion (Fig. 4.12B, gray bars) while degradation of HA by hyaluronidases significantly abrogated this response(Fig. 4.12B, teal bars). Thus, native HA is important for the induction of EGF-mediated changes in cellular behavior. Also, the more robust cellular invasion observed in epicardial cells may be the result not only of phenotypical differences but also of higher amounts of HA being produced by epicardial cells in response to EGF.

Lastly, we employed MTT assays to study the role of EGF in the rate of cell growth. As shown in figure 4.12C, cells treated with EGF proliferate at a higher rate than the untreated controls. These results are in contrast with our TGFβ2-treated cells, where we did not observe any changes in cellular proliferation. Thus, while EGF regulates embryonic cell differentiation, invasion and proliferation, TGFβ2 only modulates differentiation and invasion responses.

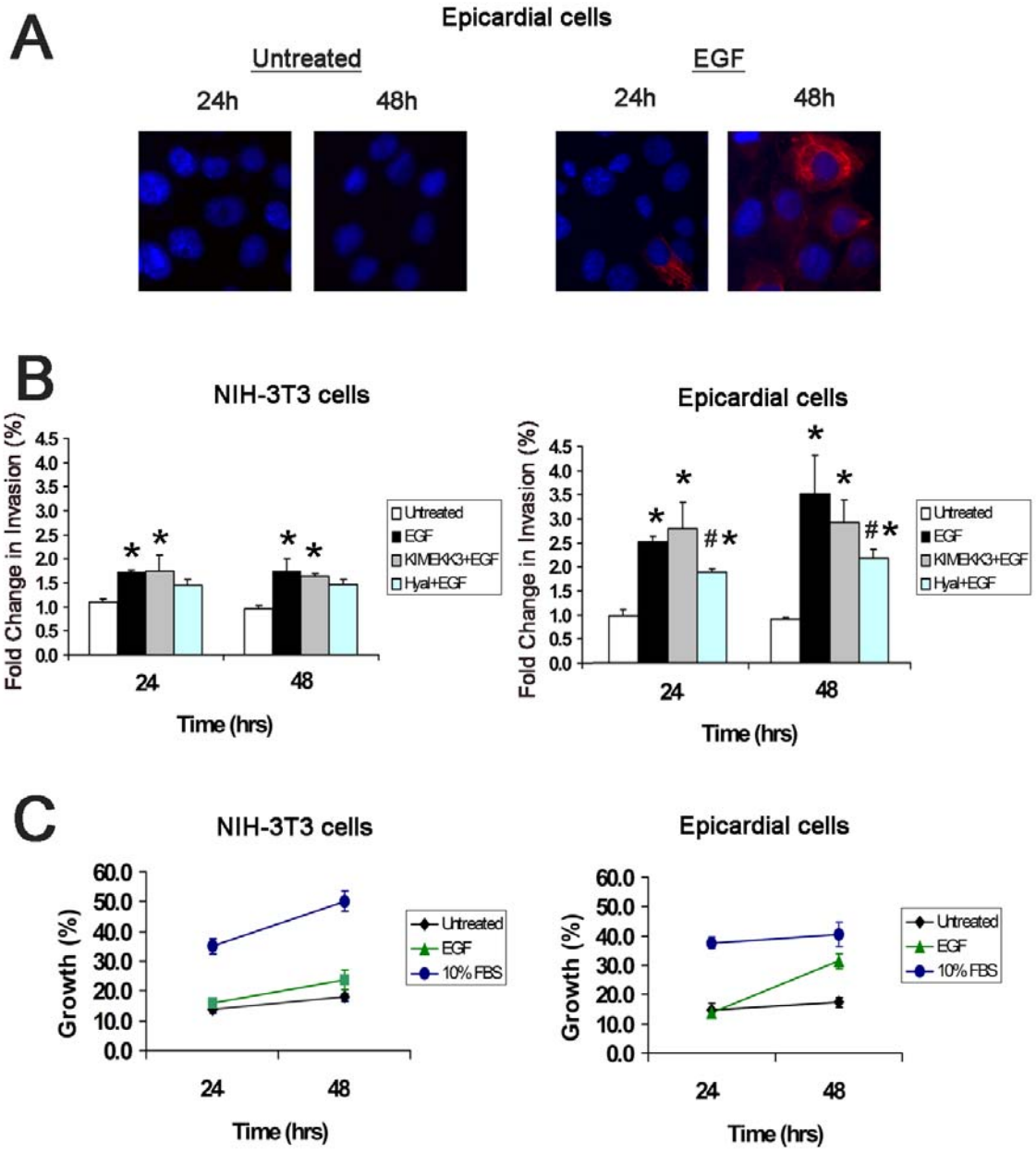

Figure 4.12. EGF modulates multiple biological responses in embryonic cells. (A) Epicardial cells were left untreated or treated with EGF for 24-48 hrs. Cells were then fixed and immunofluorescently stained with an antibody against vimentin (red) and bisbenzamide (blue) for nuclear staining. (B) Fluorescently labeled cells were stimulated with EGF in the presence or absence of KIMEKK3 or hyaluronidase and incubated for 24 or 48 hrs to allow for invasion. (C) Cell proliferation was evaluated following stimulation with EGF for 24 and 48 hrs using the MTT assay. 10% FBS was used as a positive control. *, $p<0.05$ as compared to the untreated control; #, $p<0.05$ as compared to treatment with EGF alone.

4.3. Discussion

Growth factors such as TGFβ2 and EGF ligands are highly expressed in the developing heart and a number of studies have demonstrated a prominent role for this molecule in the regulation of EMT (6, 43, 62, 94). However, many other proteins, such as Has2 (18) and members of the MAPK pathway (136, 145, 176), have also been identified as relevant for the EMT process and how these players may be interconnected remains largely unknown.

Our present study provides the first direct evidence that embryonic cells increase HA production in response to TGFβ2 and EGF and that this synthesis is important for the induction of cellular differentiation and invasion. We found that TGFβ2 is able to modulate HA synthesis via upregulation of Has2 expression through a mechanism requiring MEKK3, ERK1/2 and ERK5. On the other hand, EGF modulates Has2 expression independently of MEKK3 and also induces Has2 phosphorylation, leading to increased HA production. Both TGFβ2 and EGF induce cellular responses that dependent on the presence of native HA. These results demonstrate a novel functional relationship between growth factors and HA-mediated signals that is crucial for the orchestration of changes in embryonic cell biology. Also, our data further confirm initial evidence involving each of these molecules in the regulation of cardiovascular morphogenesis.

Despite its simple carbohydrate structure, HA has been shown to have profound effects on multiple developing organs and tissues, including the brain, heart and eyes

(75, 107, 134). HA not only provides structural support as a component of the extracellular matrix, but it also binds cell surface receptors and induces cellular responses such as differentiation and migration (14, 22, 142). Although it has been established that these responses are dependent on HA size, much controversy remains in the field in regards to whether it is the high or the lower molecular weight HA that is biologically relevant. In the context of cardiovascular development, HA oligosaccharides have been shown to induce angiogenesis but inhibit EMT (28, 123). On the other hand, high molecular weight HA promotes EMT in endocardial cushions and epicardial cells (18, 22). In this regard, our current findings are consistent with previous studies as we show that degradation of native HA by hyaluronidases impairs epicardial cell differentiation (Fig. 4.7) and significantly decreases the invasive response to TGFβ2 and EGF in NIH-3T3 and epicardial cells (Fig. 4.8 and Fig. 4.12).

MEKK3 is a mitogen activated protein kinase kinase kinase (MAP3K) and as such it participates in the transduction of extracellular signals by triggering the activation of several downstream kinases. The activation of these MAPK cascades results in a variety of responses, including proliferation, differentiation, migration and apoptosis, depending on the cellular context (24). The importance of MEKK3 in cardiovascular development has been highlighted by targeted disruption of the MEKK3 gene in mice, which leads to severe vascular and myocardial abnormalities and results in embryonic death by E11 (170). Furthermore, lack of MEKK3 kinase activity has been shown to inhibit the ability of endocardial cushions to undergo EMT while the presence of a constitutively active form of MEKK3 promotes EMT in areas of

the heart that do not normally undergo this process, such as the ventricular endocardium (145). Thus, our results further validate the role of MEKK3 in developmental EMT and also underline a previously unknown mechanism of MEKK3 activation through TGFβ2. Interestingly, constitutively active MEKK3 has also been shown to induce TGFβ2 expression (145), suggesting that a positive feedback loop may exist between these two molecules.

Among the proteins that can be activated in response to MEKK3, the MAPKs ERK1/2, p38, JNK and ERK5 have all been shown to play a role in the EMT process (20, 122, 130, 176). However, no studies have explored whether any of these molecules are specifically under the control of MEKK3 during developmental EMT. Thus, we assessed this important question and our findings demonstrate that, in epicardial cells, MEKK3 activates ERK1/2 and ERK5, but not SPK/JNK or p38, in response to TGFβ2 (Fig. 4.2). Furthermore, ERK1/2 and ERK5 activity are critical for TGFβ2 to induce HA production and promote epicardial cell differentiation and invasion. These data add to the growing evidence suggesting an important contribution for ERK5 in the development of the cardiovascular system. Disruption of the ERK5 gene in mice, for example, leads to severe and lethal defects such as abnormal heart looping and a disorganized vasculature (120). Also, although ERK1 and ERK2 knockout mice exhibit only minor non-lethal developmental defects, these proteins have been implicated in the induction of HA and the regulation of multiple cellular responses during embryogenesis (9, 19, 66, 150). Thus, ERK1/2 may have a collaborative and/or synergistic role in the transduction of developmental cues.

In contrast to what we observed in epicardial cells, MEKK3 activates ERK1/2 and p38 in NIH-3T3 cells, but in this case, only ERK1/2 appears to have a role in the regulation of Has2 and the induction of cellular responses by TGFβ2. It is worth noting that previous studies using normal adult and cancer cells have implicated JNK and p38 in TGFβ2-mediated EMT but similar experiments utilizing embryonic cells have failed to identify such connection (6, 11, 41). These observations, together with our results suggest that TGFβ2 selectively regulates MAPKs based on the specific cell type and that MAP3Ks such as MEKK3 may be more widely activated.

The present study is the first to our knowledge to reveal a novel MEKK3-dependent mechanism through which TGFβ2 regulates Has2 and its product hyaluronan. This regulation is important for the induction of embryonic cell differentiation and invasion. Furthermore, we have shown that EGF also induces Has2 expression and HA production as a mechanism to regulate cellular responses, although MEKK3 is not required in this case (Fig. 4.13). These results underscore a direct functional connection between TGFβ2, EGF and HA-mediated pathways that is critical for the orchestration of cellular changes during development.

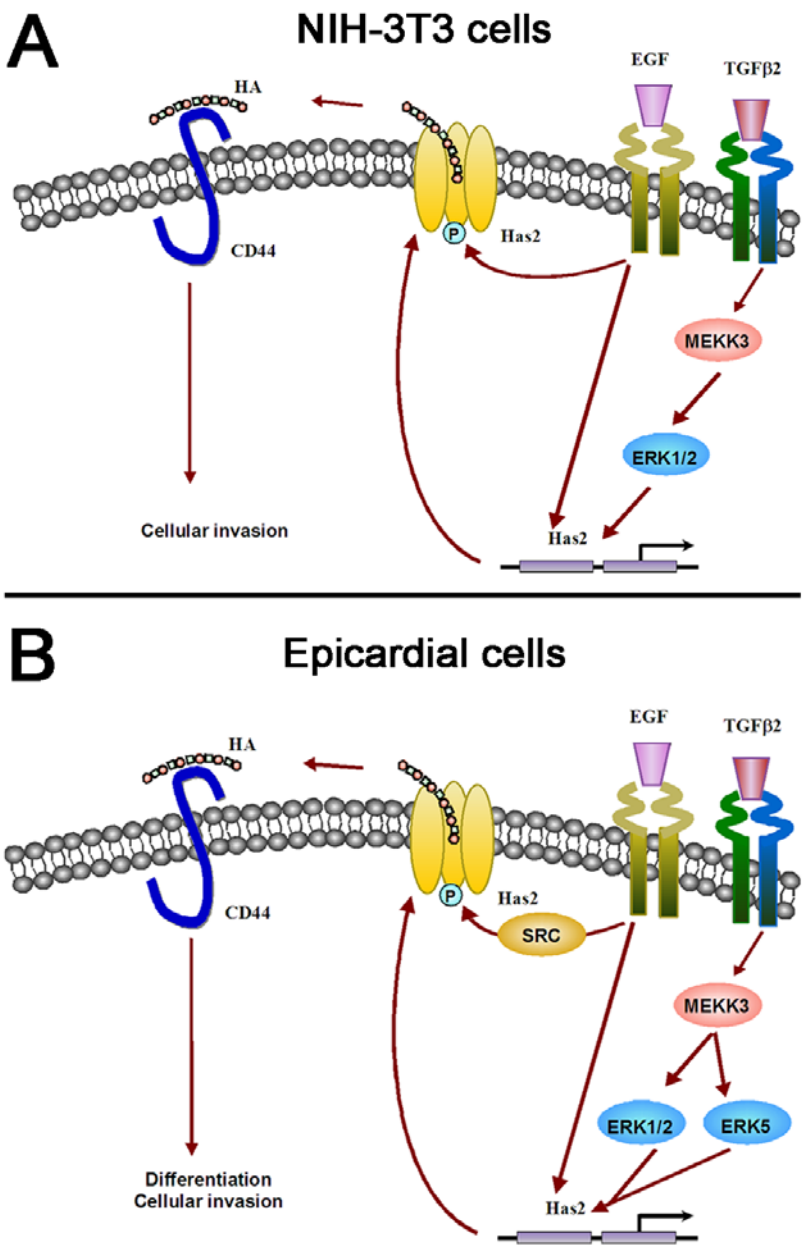

Figure 4.13. EGF and TGFβ2 regulate HA synthesis and embryonic cellular responses. (A) In NIH-3T3 cells, TGFβ2 induces MEKK3 and ERK1/2, leading to increased Has2 expression, HA production and cell invasion. On the other hand, EGF regulates Has2 expression and phosphorylation independently of MEKK3, resulting in increased HA production and cellular invasion. (B) In epicardial cells, TGFβ2 induces MEKK3, which subsequently activates ERK1/2 and ERK5, leading to increases in Has2 expression, HA synthesis and cellular differentiation and invasion. Alternatively, EGF promotes Has2 expression in a MEKK3-independent manner and Has2 phosphorylation through Src. This increases HA production and results in cellular differentiation and invasion.

4.4. Experimental Procedures

Cell culture:

NIH-3T3 cells and the inducible immortalized epicardial cell line were obtained and cultured as described in Chapter 2. All cells are of mouse origin and all treatments were performed at 37°C.

Reagents:

Human recombinant TGFβ2 and EGF were obtained from R&D Systems and used at a concentration of 2 ng/mL and 20 ng/mL, respectively. The hemagglutinin-tagged kinase-inactive MEKK3 construct (K391M) was kindly provided by Dr. Richard Vaillancourt (University of Arizona, Tucson, AZ). The ERK activation inhibitor peptide (500 nM) and p38 inhibitor (35nM) were purchased from Calbiochem (San Diego, CA). Antibodies against MEKK3, pERK5 and Has2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) while those against hemagglutinin and □-actin were from Roche Applied Science (Indianapolis, IN) and Affinity Bio Reagents (Rockford, IL), respectively. Antibodies recognizing ERK1/2, ERK5, phospho-p38, p38, phospho SPK/JNK, SPK/JNK and vimentin were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against phospho-ERK1/2 and phospho-MEKK3 were obtained from Sigma (St Louis, MO) and the Vaillancourt lab, respectively.

Measurement of HA:

Cells were seeded onto 6-well plates and subjected to various treatments. Cell culture supernatants were collected 24 and 48 hrs post treatment and HA

concentrations were determined using the Duoset ELISA development system (R&D systems) according to the manufacturer's instructions. Briefly, 96-well plates were coated with 0.5 μg/mL of recombinant human Aggrecan and incubated overnight at 25°C. Next, plates were washed, blocked with a 5% Tween-PBS solution, and incubated with HA standards or sample supernatants for 2 hrs. Plates were then washed and incubated with 0.3 μg/mL of biotinylated recombinant human Aggrecan for 2 hrs, followed by addition of horseradish peroxidase-streptavidin for 20 min. A substrate solution (H_2O_2: tetramethylbenzidine) was then added until sufficient color developed (~15 min) and the reaction was stopped with 2N H_2SO_4. The optical density of each well was determined using a microplate reader set to 450 nm with a wavelength correction of 540 nm. A linear standard curve was used to calculate HA concentrations and the data are shown as the percentage change in HA production as compared to the untreated controls.

Western Blotting:

Cells were serum starved overnight and treated with various inhibitors and/or TGFβ2 or EGF. Total cellular lysates were then resolved by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. After blocking in 3% BSA, membranes were probed with different primary and secondary antibodies. Detection was performed using Super Signal West Pico substrate (Pierce). Quantification by densitometry was performed with the ImageJ program (NIH, Bethesda, MD).

Immunofluorescence:

Cells grown and treated on glass coverslips were fixed with paraformaldehyde, permeabilized with Triton X-100, and blocked with BSA. Cells were then incubated with anti-vimentin antibody overnight and fluorescently labeled with AlexaFluor-594 secondary antibody (Molecular Probes, Eugene, OR) for 1 hour at room temperature. Fluorescent images were taken with the Leica DMLB fluorescence microscope (Leica, Bannockburn, Ill) using the Image ProPlus software (Media Cybernetics, Bethesda, Md).

Real-time PCR:

After the indicated treatments, Total RNA was isolated and quantitative real-time PCR was performed as described in Chapter 3. The gene RPS7 was used for normalization. The following primer sequences were employed: *RPS7*: AGCACGTGGTCTTCATTGCT, CTGTCAGGGTACGGCTTCTG; *Has2*: GTTGGAGGTGTTGGAGGAGA, AAAGCCATCCAGTATCTCACG; *ERK5*: TTGGTCACCACATCAAAAGC, TACGGGGTGGTGTCTTCG.

Proliferation Assays:

Cells were seeded into 96-well culture plates and routinely cultured overnight. Next, cells were serum starved for 1 hour, followed by treatment with TGFβ2 or EGF. Cell proliferation was assessed 24 and 48 hrs post treatment using the Vybrant® MTT cell proliferation assay kit (Molecular Probes) as indicated by the manufacturer.

Invasion Assays:

The ability of TGFβ2 or EGF to induce cellular invasion was determined by using a transwell chamber system, as described in Chapter 3. Briefly, fluorescently labeled cells were placed onto collagen gels casted in the upper chambers of a transwell 96-well plate. Cells were then treated with different inhibitors and/or TGFβ2 or EGF and incubated in low serum media for 24 or 48 hours. Invasion was quantified by measuring fluorescently labeled cells that moved through the collagen gels and crossed into the lower chambers of the transwell system. Fluorescence was determined at 538 nm using the Spectramax Gemini plate reader (Molecular Devices, Sunnyvale, CA).

siRNA Experiments:

Small interfering RNA (siRNA) against ERK5 and control siRNA (siRNA-A) were purchased from Santa Cruz Biotechnology. Cells grown to 50% confluence in 6-well plates were transfected with either ERK5 siRNA or control siRNA using XtremeGene siRNA transfection reagent according to the manufacturer's instructions (Roche Molecular Systems). Following transfection, cells were incubated for 48 h in a medium containing 10% FBS prior to their use in different experiments.

Statistical Analysis:

All quantitative data were analyzed using two sample Student's t tests. The data are presented as the means ± S.D. of 2 independent experiments performed in triplicate, except for invasion assays, in which each condition was analyzed in sets of 6. Differences of $P<0.05$ were considered to be significant.

CHAPTER 5

SUMMARY OF STUDIES AND FUTURE DIRECTIONS

5.1. Overview

The present dissertation focuses on Has2 and HA biology as it pertains to the formation of the cardiovascular system. The intracellular signaling mechanisms that convey Has2 and HA responses remained largely unexplored when this project was started and as such these represented an important niche in developmental research. Thus, our studies aimed at elucidating and describing novel interactions and signal transduction pathways that are relevant during organ development, especially the heart. Understanding of this molecular machinery is crucial to help uncover the underlying causes and possible treatments for pathologies such as congenital heart defects and coronary artery disease.

We performed in vitro experiments using two different cells lines: epicardial progenitor cells with an epithelial phenotype and the capacity to further differentiate; and NIH-3T3 cells, which are mesenchymal in phenotype and are a representative model of mesenchymal endocardial cushion cells. This allowed us to assess the roles of Has2 and HA in multiple stages of EMT including proliferation, differentiation and invasion and thus provided us with a more global picture of the effects of these molecules in the developing cardiovasculature. We also performed multiple

screenings and used a variety of pharmacological inhibitors to identify the specific molecules acting as intracellular mediators of HA-induced cellular responses. Furthermore, we studied how HA signals may be interconnected with other networks that are also developmentally important such as those activated by the growth factors EGF and TGFβ2.

5.2. Conclusions

Specific and detailed conclusions were provided in the results sections of each Chapter. This section presents the global conclusions of our project and the relevance of these findings to the field of HA biology.

5.2.1. HA size is an important determinant of its biological activity in embryonic cells:

The use of HA preparations of two differing molecular weights (~1 MDa and ~31 kDa) at physiological concentrations allowed us to determine the role of HA size in the regulation of embryonic cell behavior. High molecular weight HA (HMW-HA) was able to induce differentiation and invasion in epicardial cells and invasion in NIH-3T3 cells, while low molecular weight HA (LMW-HA) did not promote any significant responses. Therefore, HMW-HA, but not LMW-HA, induces changes in embryonic cell behavior independently of their phenotype. These results demonstrate that HMW-HA is the most relevant form of HA for the regulation of developmental cues and as such, HMW-HA is crucial for the proper formation of cardiovascular structures.

5.2.2. High molecular weight HA promotes embryonic cell differentiation and invasion through MEKK1-dependent pathways:

With the use of specific pharmacological inhibitors and gene silencing technology we identified key intracellular players in the transduction of HA signals. We demonstrated that, in both epicardial and NIH-3T3 cells, HMW-HA binds the cell surface receptor CD44. This induces CD44/MEKK1 association and subsequently activates downstream MAPK cascades that participate in the induction of cell differentiation and invasion. These studies are the first to elucidate how extracellular HA signals are conveyed and propagated by MAPK pathways to bring about embryonic cell responses.

5.2.3. EGF and TGFβ2 regulate embryonic cell behavior through the modulation of endogenous HA production by Has2:

Here we showed that both EGF and TGFβ2 stimulate HA production and that these increased HA levels are important for cellular responses to growth factors in embryonic cells. However, EGF and TGFβ2 utilize distinct mechanisms for HA regulation. TGFβ2 promoted HA synthesis mainly through MEKK3-mediated induction of Has2 expression. On the other hand, EGF induced Has2 expression through MEKK3-independent pathways and also regulated Has2 phosphorylation via src kinases. These studies not only establish a functional connection between growth factor signals and HA biology but also describe novel intracellular mechanisms of HA regulation. Furthermore, these findings provide a more global understanding of how

multiple signals may be integrated in various cell types to bring about the required morphogenetic changes for proper embryonic development.

We propose a model in which TGFβ2 promotes Has2 expression in an MEKK3-dependent manner while EGF induces Has2 expression and phosphorylation independently of MEKK3. These increases in Has2 activity lead to enhanced HA production and subsequent cellular differentiation and invasion. The cellular responses to HA are mediated by CD44 and MEKK1-dependent pathways in both epithelial and mesenchymal embryonic cells (Figure 5.1). These findings are specially relevant in the context of cardiac EMT as it demonstrates how a complex array of molecules interplays to regulate this process.

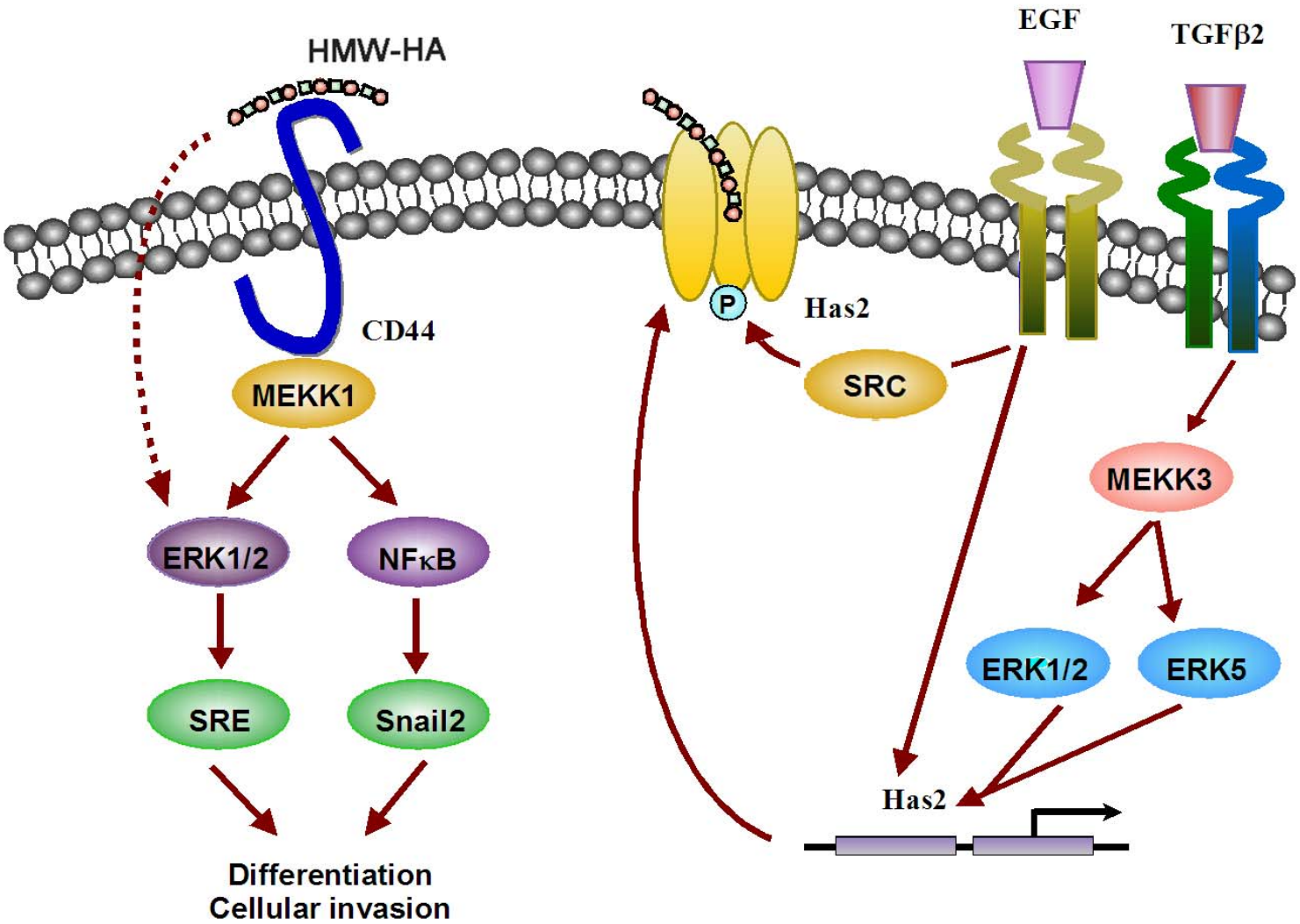

Figure 5.1. A schematic diagram of integration between the EGF, TGFβ2 and HA pathways in embryonic cells. We propose that EGF and TGFβ2 regulate Has2 activity and HA production. Increased HA levels lead to activation of MEKK1-dependent pathways and inducition of cellular resposes.

5.3 Future Studies

Our studies have elucidated several novel interactions and signaling mechanisms that are relevant in the field of hyaluronan and developmental cellular biology. However, we have also identified many other areas that require further exploration and in which significant contributions can be made. Some of the most pressing questions that remain to be answered and that stem from our current work are listed in the following sub-sections.

5.3.1 Which other molecules are controlled by HMW-HA to induce cellular responses?

We have shown that HA stimulates the intracellular signaling machinery in epicardial and fibroblast cells by interacting with the cell surface receptor CD44. However, CD44 is not solely responsible for transducing HA signals in epicardial cells, as blockade of this receptor only partially decreases HA-induced invasion and vimentin expression (Fig. 3.7). Thus, additional experiments are necessary to determine whether any of the other known HA receptors participate in the regulation of embryonic cellular responses. Furthermore, it is possible that novel HA-binding molecules on the cell surface may be identified with this research.

Our studies have also identified the transcription factor NFκB as an important activator of the Snail2 gene and a mediator of HA signals. However, it is likely that NFκB regulates more than just one gene in response to HA and this requires further exploration. A PCR array using cDNA from cells stimulated with HA, for example, may

help identify multiple HA-responsive genes, which may be activated through either the NFκB or ERK1/2 pathway.

5.3.2. What are the specific sites phosphorylated in Has2 following EGF stimulation?

We have demonstrated that EGF induces the phosphorylation of Has2 in tyrosine residues in both epicardial and fibroblast cells (Fig. 4.10). Nevertheless, the specific residues being phosphorylated within the Has2 protein structure remain unknown. One of the reasons for this is that endogenous levels of Has2 are insufficient for the mapping of phosphorylation sites through mass spectrometry. Thus, our initial approach was to create Has2 constructs for the production of Has2 recombinant protein. However, over expression of full length Has2 in mammalian, bacterial and insect cells led to cell death, which did not allow for the collection of enough Has2 protein for the identification of phosphorylation sites. To circumvent this problem, we aligned the sequences of hyaluronan synthases from multiple species using the Biology Workbench website (Fig. 5.2). With this, we identified the conserved region of the Has2 protein, where tyrosine phosphorylation is most likely to occur, and proceeded to make constructs containing just this small portion of Has2.

The Has2 conserved region was amplified by PCR from the Has2 open reading frame obtained from mouse embryos (140). The amplified fragment, which contains 10 conserved tyrosine residues, was introduced into a pcDNA4/HisMax-TOPO vector, according to the manufacturer's instructions (Invitrogen) (Fig. 5.3). This construct is for expression in mammalian cells and contains a polyhistidine region as well as an

Xpress epitope, which facilitates detection with antibodies that are specific to these regions.

Figure 5.2. Alignment of hyaluronan synthases from multiple species using Biology Workbench.

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* - single, fully conserved residue  : - conservation of strong groups
. - conservation of weak groups        - no consensus

CLUSTAL W (1.81) multiple sequence alignment

HAS2_MOUSE      ----------MHCERFLCVLRIIGTTLFGVSLLLGITAAYIVGYQFIQTDNYYFSFGLYG
HAS2_BOVIN      ----------MHCERFLCILRIIGTTLFGVSLLLGITAAYIVGYQFIQTDNYYFSFGLYG
HAS2_CHICK      ----------MYCERFICILRILGTTLFGVSLLLGITAAYIVGYQFIQTDNYYFSFGLYG
HAS2_XENLA      ----------MHCERFICILRIIGTTLFGVSLLLGISAAYIVGYQFIQTDNYYFSFGLYG
HAS3_MOUSE      ----------MPVQ-LTTALRVVGTSLFALVVLGGILAAYVTGYQFIHTEKHYLSFGLYG
HAS3_HUMAN      ----------MPVQ-LTTALRVVGTSLFALAVLGGILAAYVTGYQFIHTEKHYLSFGLYG
HAS1_MOUSE      MR-QDMPKPSEAARCCSGLARRALTIIFALLILGLMTWAYAAGVPLASDRYGLLAFGLYG
HAS1_HUMAN      MRQQDAPKPTPAACRCSGLARRVLTIAFALLILGLMTWAYAAGVPLASDRYGLLAFGLYG

HAS2_MOUSE      AFLASHLIIQSLFAFLEHRKMKKSLET-----PIKLN-----KTVALCIAAYQEDPDYLR
HAS2_BOVIN      AFLASHLIIQSLFAFLEHRKMKKSLET-----PIKLN-----KTVALCIAAYQEDPDYLR
HAS2_CHICK      AILASHLIIQSLFAYLEHRKMKRSLET-----PIKLN-----KTVALCIAAYQEDPDYLR
HAS2_XENLA      AILALHLIIQSLFAFLEHRKMKRSLET-----PIKLN-----KSVALCIAAYQEDEDYLR
HAS3_MOUSE      AILGLHLLIQSLFAFLEHRRMRR-AGR-----PLKLHCSQRSRSVALCIAAYQEDPEYLR
HAS3_HUMAN      AILGLHLLIQSLFAFLEHRRMRR-AGQ-----ALKLPSPRRG-SVALCIAAYQEDPDYLR
HAS1_MOUSE      AFLSAHLVAQSLFAYLEHRRVAAAARRSLAKGPLDAAT---ARSVALTISAYQEDPAYLR
HAS1_HUMAN      AFLSAHLVAQSLFAYLEHRRVAAAAR-----GPLDAAT---ARSVALTISAYQEDPAYLR

HAS2_MOUSE      KCLQSVKRLTYP--GIKVVMVIDGNSDDDLYMMDIFSEVIG-RDKSATYIWKNNFHE---
HAS2_BOVIN      KCLQSVKRLTYP--GIKVVMVIDGNSEDDLYMMDIFSEVMG-RDKSATYIWKNNYHV---
HAS2_CHICK      KCLLSVKRLTYP--GIKVVMVIDGNSEDDVYMMDIFTEIMG-RDKSATYIWSNNFHD---
HAS2_XENLA      KCLLSVKRLTYP--GMKVIMVIDGNSDDDLYMMNIFREIMG-NDSCATYVWKNNFHM---
HAS3_MOUSE      KCLRSAQRIAFP--NLKVVMVVDGNRQEDTYMLDIFHEVLGGTEQAGFFVWRSNFHE---
HAS3_HUMAN      KCLRSAQRISFP--DLKVVMVVDGNRQEDAYMLDIFHEVLGGTEQAGFFVWRSNFHE---
HAS1_MOUSE      QCLTSARALLYPHTRLRVLMVVDGNRAEDLYMVDMFREVFA-DEDPATYVWDGNYHQPWE
HAS1_HUMAN      QCLASARALLYPRARLRVLMVVDGNRAEDLYMVDMFREVFA-DEDPATYVWDGNYHQPWE

HAS2_MOUSE      -------KGPGETEESHKE--SSQHVTQLVLSNKSICIMQKWGGKREVMYTAFRALGRSV
HAS2_BOVIN      -------KGPGETDESHKE--SSQHVTQLVLSNKSICTMQKWGGKREVMYTAFRALGRSV
HAS2_CHICK      -------KGPGETEESHRE--SMQHVSQLVLSNKSVCIMQKWGGKREVMYTAFKALGEAW
HAS2_XENLA      -------KGPNETDETHRE--SMQHVTQMVLSNRNVCIMQKWNGKREVMYTAFKALGRSV
HAS3_MOUSE      -------AGEGETEASLQE--GMERVRAVVWASTFSCIMQKWGGKREVMYTAFKALGNSV
HAS3_HUMAN      -------AGEGETEASLQE--GMDRVRDVVRASTFSCIMQKWGGKREVMYTAFKALGDSV
HAS1_MOUSE      PAEATGAVGEGAYREVEAEDPGRLAVEALVRTRRCVCVAQRWGGKREVMYTAFKALGDSV
HAS1_HUMAN      PAAA-GAVGAGAYREVEAEDPGRLAVEALVRTRRCVCVAQRWGGKREVMYTAFKALGDSV

HAS2_MOUSE      DYVQVCDSDTMLDPASSVEMVKVLEEDPMVGGVGGDVQILNKYDSWISFLSSVRYWMAFN
HAS2_BOVIN      DYVQVCDSDTMLDPASSVEMVKVLEEDPMVGGVGGDVQILNKYDSWISFLSSVRYWMAFN
HAS2_CHICK      NYVQVCDSDTMLDPASSVEMVKVLEEDPMVGGVGGDVQILNKYDSWISFLSSVRYWMAFN
HAS2_XENLA      DYVQVCDSDTVLDPASSVEMVKVLEEDIMVGGVGGDVQILNKYDSWISFLSSVRYWMAFN
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HAS3_MOUSE      DYIQVCDSDTVLDPACTIEMLRVLEEDPQVGGVGGDVQILNKYDSWISFLSSVRYWMAFN
HAS3_HUMAN      DYIQVCDSDTVLDPACTIEMLRVLEEDPQVGGVGGDVQILNKYDSWISFLSSVRYWMAFN
HAS1_MOUSE      DYVQVCDSDTRLDPMALLELVRVLDEDPRVGAVGGDVRILNPLDSWVSFLSSLRYWVAFN
HAS1_HUMAN      DYVQVCDSDTRLDPMALLELVRVLDEDPRVGAVGGDVRILNPLDSWVSFLSSLRYWVAFN

HAS2_MOUSE      IERACQSYFGCVQCISGPLGMYRNSLLHEFVEDWYNQEFMGNQCSFGDDRHLTNRVLSLG
HAS2_BOVIN      IERACQSYFGCVQCISGPLGMYRNSLLHEFVEDWYNQEFMGSQCSFGDDRHLTNRVLSLG
HAS2_CHICK      IERACQSYFGCVQCISGPLGMYRNSLLHEFVEDWYNQEFMGSQCSFGDDRHLTNRVLSLG
HAS2_XENLA      IERACQSYFGCVQCISGPLGMYRNSLLHEFIEDWYNQEFLGSQCSFGDDRHLTNRVLSLG
HAS3_MOUSE      VERACQSYFGCVQCISGPLGMYRNSLLQQFLEDWYHQKFLGSKCSFGDDRHLTNRVLSLG
HAS3_HUMAN      VERACQSYFGCVQCISGPLGMYRNSLLQQFLEDWYHQKFLGSKCSFGDDRHLTNRVLSLG
HAS1_MOUSE      VERACQSYFHCVSCISGPLGLYRNNLLQQFLEAWYNQKFLGTHCTFGDDRHLTNRMLSMG
HAS1_HUMAN      VERACQSYFHCVSCISGPLGLYRNNLLQQFLEAWYNQKFLGTHCTFGDDRHLTNRMLSMG
                      *** **.*******:***.**: *:* **:* *:* :*:**********:**:*

HAS2_MOUSE      YATKYTARSKCLTETPIEYLRWLNQQTRWSKSYFREWLYNAMWFHKHHLWMTYEAVITGF
HAS2_BOVIN      YATKYTARSKCLTETPIEYLRWLNQQTRWSKSYFREWLYNAMWFHKHHLWMTYEAVITGF
HAS2_CHICK      YATKYTARSKCLTETPIEYLRWLNQQTRWSKSYFREWLYNAMWFHKHHLWMTYEAVITGF
HAS2_XENLA      YATKYTARSKCLTETPTEYLRWLNQQTRWSKSYFREWLYNSLWFHKHHLWMTYEAVITGF
HAS3_MOUSE      YRTKYTARSKCLTETPTRYLRWLNQQTRWSKSYFREWLYNSLWFHKHHLWMTYESVVTGF
HAS3_HUMAN      YRTKYTARSKCLTETPTKYLRWLNQQTRWSKSYFREWLYNSLWFHKHHLWMTYESVVTGF
HAS1_MOUSE      YATKYTSRSRCYSETPSSFLRWLSQQTRWSKSYFREWLYNALWWHRHHAWMTYEAVVSGL
HAS1_HUMAN      YATKYTSRSRCYSETPSSFLRWLSQQTRWSKSYFREWLYNALWWHRHHAWMTYEAVVSGL
                : ****:**:* :***  :****.****************::*:*:** *****:*::*:

HAS2_MOUSE      FPFFLIATVIQLFYRGKIWNILLFLLTVQLVGLIKSSFASCLRGNIVMVFMSLYSVLYMS
HAS2_BOVIN      FPFFLIATVIQLFYRGKIWNTLLFLLTVQLVGLIKSSFASCLRGNIVMVFMSLYSVLYMS
HAS2_CHICK      FPFFLIATVIQLFYRGKIWNILLFLLTVQLVGLIKSSFASFLRGNIVMVFMSLYSVLYMS
HAS2_XENLA      FPFFLIATVIQLFYRGRIWNILLFLLTVQLVGLIKSSFASALRGNIVMVFMSFYSVLYMS
HAS3_MOUSE      FPFFLIATVIQLFYRGRIWNILLFLLTVQLVGIIKATYACFLRGNAEMIFMSLYSLLYMS
HAS3_HUMAN      FPFFLIATVIQLFYRGRIWNILLFLLTVQLVGIIKATYACFLRGNAEMIFMSLYSLLYMS
HAS1_MOUSE      FPFFVAATVLRLFYAGRPWALLWVLLCVQGVALAKAAFAAWLRGCVRMVLLSLYAPLYMC
HAS1_HUMAN      FPFFVAATVLRLFYAGRPWALLWVLLCVQGVALAKAAFAAWLRGCLRMVLLSLYAPLYMC
                ****: ***::*** *: *  * .** ** *.: *:::*. ***   *:::*:*: ***

HAS2_MOUSE      SLLPAKMFAIATINKAGWGTSGRKTIVVNFIGLIPVSVWFTILLGGVIFTIYKESKKPFS
HAS2_BOVIN      SLLPAKMFAIATINKAGWGTSGRKTIVVNFIGLIPVSVWFTILLGGVIFTIYKESKKPFS
HAS2_CHICK      SLLPAKMFAIATINKAGWGTSGRKTIVVNFIGLIPVSIWFTILLGRVIFTIYKESKKPFS
HAS2_XENLA      SLLPAKMFAIATINKAGWGTSGRKTIVVNFIGLIPITVWFTILLGGVCYTIWRETKKPFS
HAS3_MOUSE      SLLPAKIFAIATINKSGWGTSGRKTIVVNFIGLIPVSIWVAVLLGGLAYTAY--CQDLFS
HAS3_HUMAN      SLLPAKIFAIATINKSGWGTSGRKTIVVNFIGLIPVSIWVAVLLGGLAYTAY--CQDLFS
HAS1_MOUSE      GLLPAKFLALVTMNQSGWGTSGRKKLAANYVPVLPLALWALLLLGGLARSVAQEARADWS
HAS1_HUMAN      GLLPAKFLALVTMNQSGWGTSGRRKLAANYVPLLPLALWALLLLGGLVRSVAHEARADWS
                .*****::*: *:*::

HAS2_MOUSE      ----ESKQTVLIVGTLIYACYWVMLLTLYVVLIN-KCGRRKKGQQYDMVLDV-
HAS2_BOVIN      ----ESKQTVLIVGTLLYACYWVMLLTLYVVLIN-KCGRRKKGQQYDMVLDV-
HAS2_CHICK      ----ESKTTVLVIGTILYACYWVLLLTLYLVLIT-KCGRRKKEQHYDMVLDV-
HAS2_XENLA      ----ESEKIVLAVGAILYACYWVMLLTMYVSLVM-KCGRRRKEPQHDLVLA--
HAS3_MOUSE      ----ETELAFLVSGAILYGCYWVALLMLYLAIIARRCG--KKPEQYSLAFAEV
HAS3_HUMAN      ----ETELAFLVSGAILYGCYWVALLMLYLAIIARRCG--KKPEQYSLAFAEV
HAS1_MOUSE      GPSRAAEAYHLAAGAGAYVAYWVVMLTIYWVGVRRLCR--RRSGGYRVQV---
HAS1_HUMAN      GPSRAAEAYHLAAGAGAYVGYWVAMLTLYWVGVRRLCR--RRTGGYRVQV---
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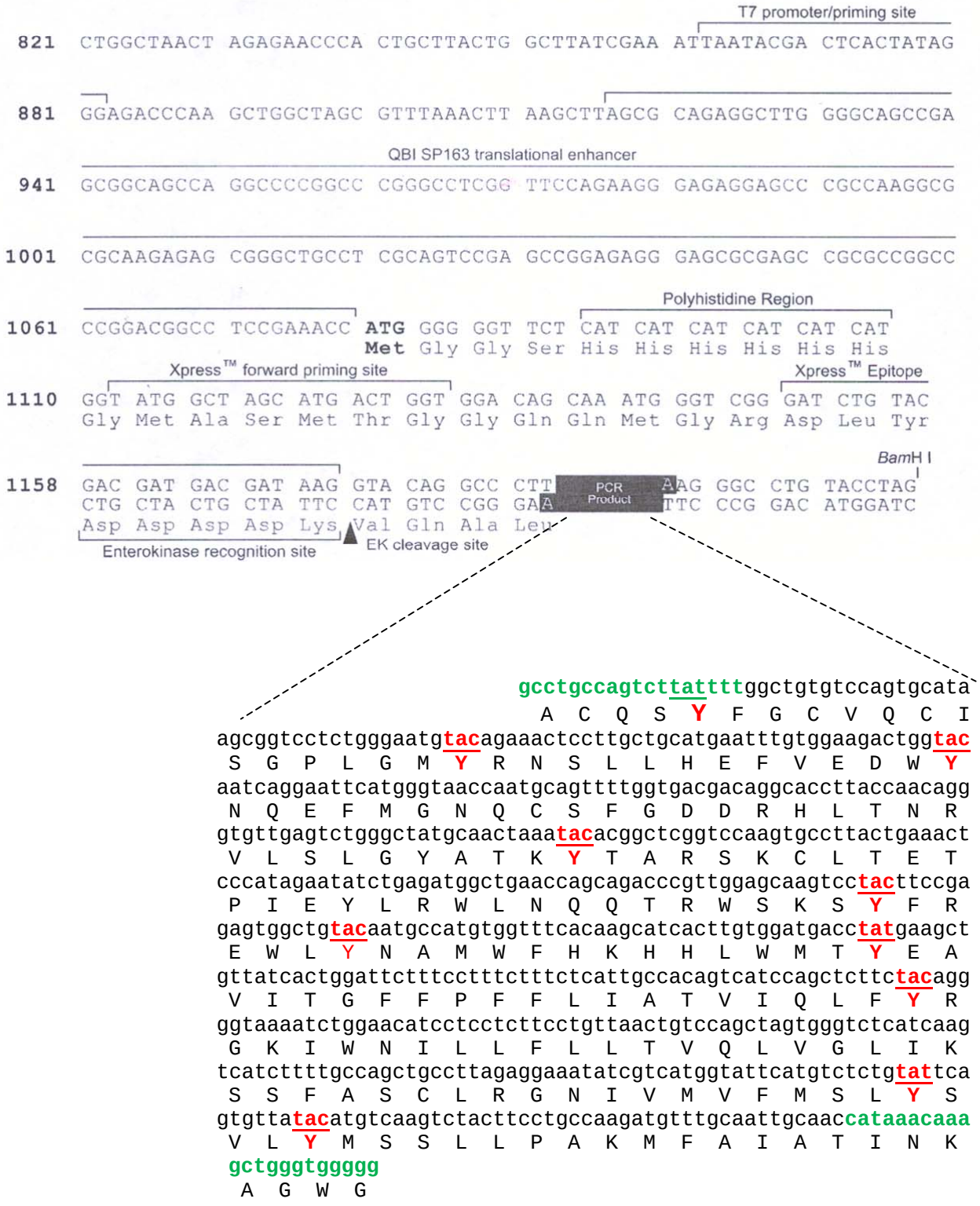

Figure 5.3. Cloning of the conserved region of Has2 into a mammalian vector. The conserved segment of Has2 was amplified using specific primers for this region (shown in green) and then inserted into a pcDNA4/HisMax-TOPO vector.

To introduce the Has2T construct, Human Embryonic Kidney (HEK) cells were infected using an adenovirus carrier conjugated with green fluorescent protein (GFP). HEK cells were chosen for this procedure because they are easier to transfect and less sensitive than either NIH-3T3 or epicardial cells and their protein yield is much higher. As shown in figure 5.3A, green fluorescence was observed after a 30 hr incubation period only in cells infected with Has2T-AdGFP.

Expression of the Has2T construct was verified by Western Blot using the anti-Xpress antibody, which detects the Xpress epitope present in the pcDNA4/HisMax-TOPO vector. We anticipate that overexpression of this construct in mammalian cells, followed by treatments with EGF, will allow for the identification of specific tyrosine phosphorylation sites through mass spectometry. If this approach does not work, an alternative procedure will be to introduce the Has2 sequence into an inducible construct, which will allow for the expression of Has2 only for shorts periods of time. This may prevent the cell death observed with prolonged recombinant Has2 expression.

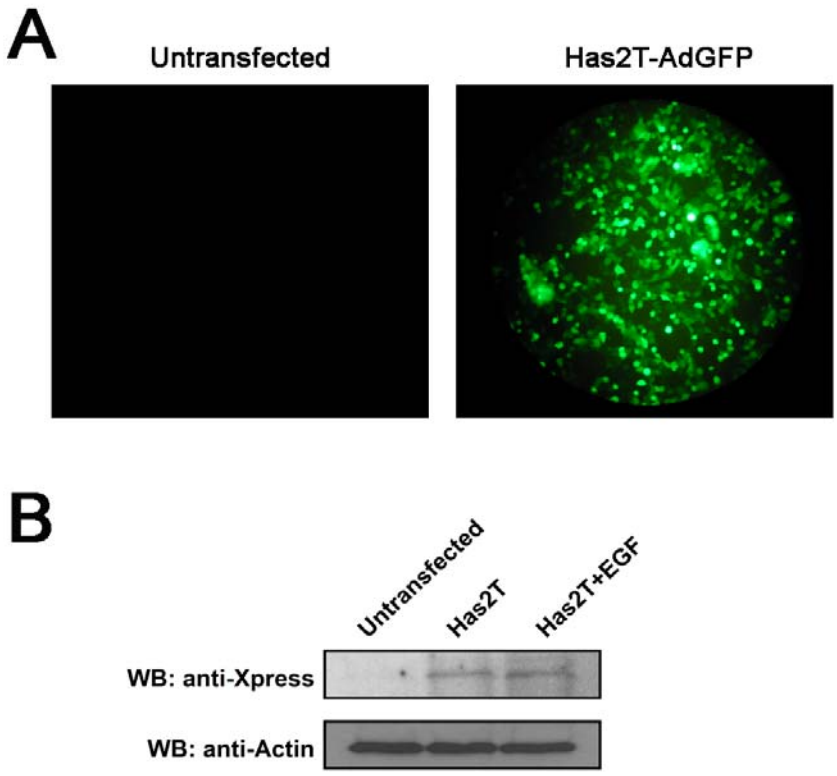

Figure 5.4. Has2T construct expression in mammalian cells. (A) A construct containing the conserved region of Has2 was introduced into HEK cells by incubation with Ad-GFP for 30hrs. (B) HEK cells were left untrastected, transfected with Has2T or transfected with Has2T followed by stimulation with EGF for 30 min. Lysates were subjected to WB with anti-Xpress antibody and anti-actin antibody as a loading control.

Another tool that we have created for future experiments consists of a construct for expression in bacterial cells (Has2T-b). This construct is composed of the pRSET vector (Invitrogen) together with the conserved region of Has2 (Fig. 5.5). We created this bacterial construct as an alternative to the mammalian one because bacterial cells grow at a faster rate and may be less sensitive to overexpression of Has2. Thus, we anticipate that this procedure will allow for sufficient production and isolation of Has2T-b recombinant protein to perform in vitro kinase assays. With this approach we

expect to identify molecules that directly bind and phosphorylate Has2 in response to EGF stimulation.

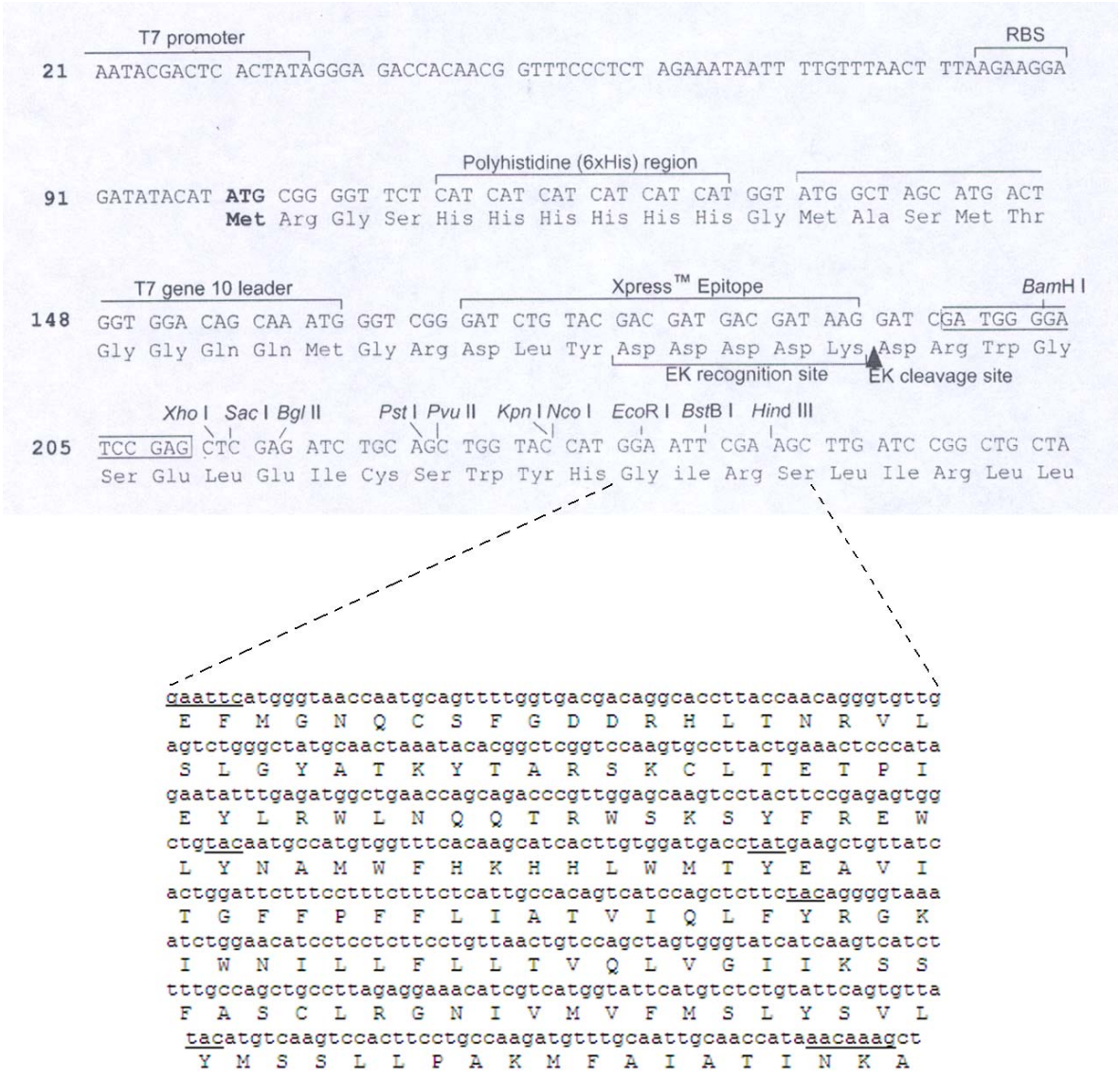

Figure 5.5. Cloning of the conserved region of Has2 into a bacterial vector. The conserved segment of Has2 was cloned into the bacterial vector pRSET through ligation at the EcoRI and HindIII sites.

5.3.3. How does EGF regulate Has2 expression?

Our results show that EGF induces Has2 expression (Fig 4.9) in both epithelial and mesenchymal cells. However, the specific intracellular molecules that participate in the regulation of Has2 by EGF have not been identified. We have demonstrated that EGF and TGFβ2 act through different mechanisms as MEKK3 and its downstream effectors are important for TGFβ2-induced increases in Has2 but do not appear to be revelant for EGF signaling. Nevertheless, further studies are necessary to establish whether EGF and TGFβ2 act simultaneously, but through distinct pathways, or whether their regulation of Has2 is spatiotemporally different.

In summary, our current research has helped decipher previously unknown intracellular effectors that participate in the interconnection of growth factor and hyaluronan signals in embryonic cells during EMT. Due to the relevance of EGF, TGFβ2 and HA for proper cardiovascular development, our findings are an important contribution to our understanding of the molecular events that direct normal cardiac morphogenesis. Furthermore, our initial studies have led to the formulation of many other scientific questions whose answers will expand the knowledge of signaling molecules with crucial developmental functions and may ultimately help improve diagnosis and repair strategies for cardiovascular diseases.

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