ROLE OF CONNEXIN 43 IN ENDOTHELIAL CELL-INDUCED MURAL CELL DIFFERENTIATION

by

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DEDICATION

To my wife Miriam and to my parents, Irina and Nikolai, for their love, support and confidence.

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ABSTRACT

Objective: Endothelial cell (EC)-induced mesenchymal cell (MC) differentiation toward a mural cell phenotype requires transforming growth factor beta (TGF-β), cell contact and connexin 43 (Cx43)- or Cx45- heterocellular gap junction intercellular communication (GJIC). However, the identity of the communicated signal, the features of Cx43 required, and the possible regulatory mechanisms have not been elucidated and were investigated herein.

Methods & Results: To determine whether channel functionality and the major regulatory domain (the carboxyl terminus, CT) of connexin Cx43 are necessary to support EC-induced differentiation, Cx43 deficient MCs (incapable of undergoing EC-induced mural cell differentiation without re-expression of Cx43 or Cx45) were transduced with wild-type (Cx43wt), channel dead, or truncated (Cx43tr-residues 258-382 deleted) versions of Cx43 and their ability to support EC-induced differentiation was assessed. Our data indicate that both channel functionality and presence of the CT domain are both necessary for EC-induced mural cell differentiation. Moreover, expression of Cx40 did not restore ability of MCs to undergo EC-induced mural cell differentiation, despite supporting GJIC.

To determine whether (and which) specific regulatory sites in the carboxyl terminus are necessary for EC-induced mural cell differentiation, constructs of Cx43 with serine to alanine substitutions at the mitogen activated protein kinase (MAPK) or protein kinase C (PKC) target sites were introduced into Cx43 deficient MCs and their ability to undergo EC-induced differentiation was tested. The data indicated that the MAPK targeted serines (S255,279,2982) are necessary, while the PKC targeted serine (S368) is dispensable, for

this process. To determine whether calcium ions might be the messengers communicated between ECs and MCs, we investigated whether elevation in EC free intracellular calcium concentration (with ionomycin treatment) can replace Cx43-mediated GJIC, activate TGF-β and induce differentiation.

Conclusions: Channel functionality, CT domain and the MAPK target sites in Cx43 are all necessary, and neither alone is sufficient, for Cx43-mediated, EC-induced mural cell differentiation. Unlike Cx43, Cx40 is not capable of supporting EC-induced differentiation, despite supporting GJIC. Calcium is unlikely to be the messenger critical to TGF-β activation during EC-induced differentiation, but similar signaling pathways can be initiated. Taken together, these data support a role for connexins in EC-induced differentiation that is complex and goes beyond that of a simple conduit.

DESCRIPTION OF FORMAT

This dissertation includes three main chapters. Chapter 1 consists of the introduction and background sections that summarize the relevance of the work and provide the information necessary to familiarize the reader with the relevant signaling elements. Chapter 2 consists of a brief summary of the specific aims and experimental outcomes. Chapter 2 is followed by three appendices (labeled A, B, and C) that contain the studies addressing Specific Aims 1,2 and 3, respectively. The final chapter, Chapter 3, provides an overall summary and some possible future directions.

I was the primary author for the manuscript investigating Specific Aim 1 and performed all of the experimental work, data analysis, and much of the manuscript preparation. It should be noted, however, that the studies investigating Specific Aims 2 and 3 (appendices B and C) were performed in collaboration with several colleagues. Thus, I did not perform all of the experiments described in those manuscripts, but I was responsible for a significant portion of the effort. My specific contributions to the manuscript investigating Specific Aim 2 consisted of generating the positive and negative control data, as well as the data associated with testing whether all of three of the Cx43 mitogen activated protein kinase-targeted serines at positions 255,279 and 282 are necessary for EC-induced mural cell differentiation. In addition, I was involved in the analysis and interpretation of the data as well as preparation of the manuscript.

My main contributions to the study investigating Specific Aim 3 consisted of exploring the transient elevations in free intracellular calcium ion concentration that occurred in endothelial and mesenchymal cells in the absence and presence of Cx43 and ionomycin treatment. In addition, I generated the data describing the expression of activin-like

kinase (ALK) receptors in the mesenchymal cell lines and activation of TGF-β signaling pathways. I was also involved in much of the data analysis/ interpretation and preparation of the manuscript. Thus, the primary authorship is shared between Dr. David Kurjiaka and my self.

CHAPTER 1: INTRODUCTION AND BACKGROUND

INTRODUCTION

A circulatory system is of critical importance in multicellular organisms beyond a size where diffusion alone is sufficient to mediate gas exchange in all cells. However, the utility of the circulatory system stretches beyond its role as a gas exchange vehicle. Because it has access to all tissues, evolutionary advantage has been conferred to those organisms utilizing the circulation for systemic trafficking of a variety of substances. In vertebrates, and most notably man, the circulatory system is utilized to carry anything from whole immune cells to signaling molecules and is intimately involved in most homeostatic and disease processes. It should, therefore, come as no surprise that the circulatory system (with its main cardiac and vascular components) has always been the subject of much interest, both scientific and not. The study of cardiovascular physiology is inextricably linked to modern medicine because all disease has cellular basis and all cells in the body depend on the circulatory system, albeit to a different extent. Thus, every sub-discipline of pathology has at least some level of vascular involvement. Although recent technological advances have revolutionized cardiovascular research, complete understanding is impractical due to: 1) the sheer complexity of the system and its interactions with all other tissues and 2) the dynamic nature of its structure and function. Nevertheless, meaningful progress that is relevant to medical advances can be achieved through the use of in-vitro and in-vivo models of vascular cell or tissue behavior.

For the purposes of convenience one can categorize diseases with vascular involvement into diseases of vascular insufficiency, excess, or abnormality. Examples of diseases

classified under vascular insufficiency are ischemic diseases, where the patient can benefit from increased local vascular density and perfusion. In contrast, diseases such as vascularized neoplastic growths and hemangiomas can be classified under diseases with vascular excess, and are situations where limiting vascular growth is therapeutically beneficial. Lastly, diseases categorized as vascular abnormality may include diseases where the vascular development was altered, resulting in sub-optimal vascular function, or diseases where the vasculature formed normally, but was altered in the course of a pathology. Although there is certainly overlap between these groups, the utility of this categorization scheme lies in the ability to delineate whether the vascular involvement depends on the proliferation or differentiation state of the vascular cells and to aid in directing the experimental design (and model selection) toward a clinically relevant set of conclusions.

The broad goal of the present work is to contribute to the body of knowledge supporting our understanding of blood vessel formation and development. More specifically, it is aimed at determining the role that connexin proteins play in the formation of the vascular wall, a component necessary for the regulation of blood flow, blood pressure, and vessel stability. The model system chosen for this work is an in-vitro system utilizing standard cell culture techniques, the advantages of which consist of exclusion of complexity contributed by tissue structure and immune responses as well as relative ease of manipulating connexin expression in any one particular cell type. As is the case with any model system, there are some limitations as well. Most of the limitations associated with this approach stem from concerns about how well data generated using an in-vitro model could be extrapolated to intact organisms. Although this is certainly a valid concern, we

are convinced of the validity of this model system because the basic interactions between the cell types studied were found to be similar in the in-vivo setting[1-3].

The following background section is intended to familiarize the reader with the general aspects of vascular development, the role of Cx proteins in the process and TGF-β signaling, all of which are relevant to the appended studies.

BACKGROUND

Initial Stages of Vascular Development

Initial stages of blood vessel development are governed by vasculogenesis, a process during which endothelial cells are generated by differentiation from multipotent precursors. Multipotent precursor cells, termed angioblasts, congregate to form clusters known as blood islands. The outermost cells differentiate into endothelial cells and cells of the inner mass differentiate into the cellular components of blood[4]. Endothelial cells proliferate and form a highly branched network structure, which acquires a lumen. This process defines the primary vascular plexus that serves as the initial blueprint of the vascular system[4]. Further growth of blood vessels proceeds mainly, although not exclusively, through the process of angiogenesis, the growth of new vessels from existing ones by proliferation and migration of endothelial cells. A simplified schematic representation of this process is depicted in Figure 1. As increases in embryonic tissue mass and metabolic demand outpace the nutrient and oxygen supply, tissues secrete growth factors that activate local endothelial cells and vascular growth is augmented[5]. Fairly early on in the developmental process, the arterial and venous sides of the circulation are specified[6] and subsequently the vessel wall is acquired. Endothelial cell tubes secrete platelet-derived growth factor (PDGF), which acts as a chemoattractant and mitogen to recruit mural cell precursors[1,2] mainly, though not exclusively, from multiple mesenchymal sources [7]. Precursor cells migrate toward endothelial cell tubes, interact directly with endothelial cells and ultimately undergo TGF-β -dependent mural cell differentiation that involves the upregulation of mural cell- specific genes. The interaction of endothelial and mesenchymal cells necessary for mural cell differentiation

is critically dependent on the presence of specific connexin isoforms (Cx43 or Cx45) and direct cell contact[3,8]. It is important to note that unlike endothelial cells, which mostly share a common origin, mural cells (smooth muscle cells and pericytes alike) are mostly recruited mesenchyme, a non-fully committed embryonic connective tissue that is present in many developing systems. However, in some settings the vascular wall is formed by differentiation from a sub-population of neural crest cells that migrate and form non-neural tissues[7,9]. Thus, mural cells found in circulatory beds of different organs do not necessarily share a common lineage. Consequently, vessel wall formation in different vascular beds might not always utilize exactly the same signaling mechanisms.

It is difficult to overstate the importance of spatiotemporal specificity of intercellular signaling during blood vessel development. That Cx expression in endothelial cells, mural cells, and their respective precursors is critical for proper blood vessel formation is evident from a number of studies abolishing various aspects of intercellular signaling[10]. In general, signaling between vascular cells can be mediated by interaction of surface adhesion proteins, direct cytoplasmic coupling via gap junctions, or soluble factors acting in paracrine or autocrine manner[11]. Exhaustive discussion of all known signaling pathways is beyond the scope of this work, but a discussion of the ones most relevant to the interaction of endothelial cells with mesenchymal cells is included herein.

Gap Junctions and Connexins

Gap junctions were first identified by electron microscopy as morphological regions of distinct intermembrane space narrowings[12-14] . The twenty-angstrom intermembrane gaps readily observed in many tissues allowed gap junctions to be morphologically, and later,

functionally[15] differentiated from tight junctions. These structures were later found in numerous tissues and described as aggregates of intercellular channels (gap junction channels) that directly connect the cytoplasm regions of adjacent cells. More recent investigations have highlighted critical roles for gap junctions and their components in a vast array of physiological and pathological processes, bringing these structures to the forefront of biological research.

I. Gap Junctions- Nomenclature

Gap junction channels are formed by the docking of hemichannels, termed connexons, integrated into the apposed membranes of adjacent cells. Each hemichannel is composed of six connexins (Cx), peptides belonging to a family of structurally related transmembrane proteins. The composition of gap junction channels may vary depending on the connexons of which it is comprised. Connexons comprised of identical connexin sub-units are referred to as homomeric, whereas those comprised of two or more connexin isotypes are termed heteromeric. Similarly, gap junction channels composed of identical connexons are referred to as homotypic, whereas those composed of two different connexons are termed heterotypic. Lastly, gap junction channels are typically found in as large aggregates termed “gap junction plaques”.

II. Gap Junctions: Structure-Function Relationships

The basic structure of gap junction channels can be described as a central hydrophilic pore with a limiting diameter of approximately one kilodalton (1kDa), allowing them to function as electrical and molecular conduits. Although substances with a hydrated radius smaller then the pore’s limiting diameter should theoretically have the ability to freely traverse the channel according to their concentration or electrical gradients, this is not

always the case. Gap junction channels have been shown to display some connexin specific permeability and can be gated to various states of closure (complete or partial) by transjunctional voltage gradients, hydrogen ion concentration changes, or post-translational modifications[16-20]. The possible permeability, conductance, and gating mechanisms are dictated by the connexin composition (and thus by the primary sequence of the component connexins). Additionally regulation of channel behavior can be cell specific due to the presence/absence or activity of regulatory molecules.

Although support of intercellular communication is the most appreciated function of gap junction channels, recent studies have uncovered a large number of concurrent functions. It has been demonstrated that hemichannels can, upon stimulus, open and allow transmembrane diffusion of ions and small molecules according their concentration gradients[21]. In addition, gap junctions co-localize with a large array of intracellular proteins seemingly not involved in modulating the communication properties of the junction (for review of Cx interacting proteins see: [22]). Thus, at least in some cases, the connexin dependent modulation of cell function (behavior) can be channel independent.

III. Connexin Structure and Nomenclature

Two nomenclatures are typically used in describing connexins. The more frequently used nomenclature designates connexins according to their apparent electrophoretic mobility (in kilodaltons (kDa)) and species of origin. Under this nomenclature the murine gap junction protein of approximately 43 kDa is designated mCx43. Under the alternative nomenclature connexins are designated into several subgroups (alpha, beta, gamma, or delta) according to their sequence similarity and length of the cytoplasmic domains. Additionally, they are also given a number according to the order in which they were

discovered. Under this nomenclature mCx43, the first alpha connexin to be discovered, is designated Gja1 (gap junction protein alpha1)[23].

Connexins share a similar overall structure and some of their features are well conserved, however there are considerable differences in some domains. All members of the connexin family have four transmembrane domains (M1-M4), an intracellular amino terminus (NT), intracellular carboxy terminus (CT) and a cytoplasmic loop (CL, between the second and third transmembrane domains). There are also two extracellular loops (EL1 and EL2), one connecting the first and second transmembrane domains and one connecting the third and fourth transmembrane domains.

While the primary sequence of the transmembrane domains is rather well conserved among different connexin isoforms, the amino and carboxy termini sequences can vary considerably. For example, connexin 43 has a rather large CT domain that contains a number of possible phosphorylation sites that can be targeted by regulatory kinases and other interacting proteins[22,24]. In contrast, Cx26 has only a very short (< 20 amino acids) CT domain[25]. The functional consequences of such structural dissimilarities have been at the forefront of gap junction research and are believed to be the basis for different conductance and permeability sub-states that gap junction channels exhibit.

Connexins in the Arterial Vasculature

Connexin proteins and communicating gap junctions can be detected in all vascular cell types and their expression is critical for the proper function and development of the cardiovascular system. Although over twenty different connexin isoforms have been identified to date, only four (Cx43, Cx40, Cx45 and Cx37) are found in the arterial

vasculature, where they exhibit some degree of spatial and temporal specificity. Vascular connexin expression, localization, or function can also be altered in settings of injury and disease[26,27], suggesting a role for these proteins in adaptive responses as well as homeostatic balance.

I. Expression

Quiescent endothelial cells lining large and mature blood vessels predominantly express Cx40 and Cx37, whereas proliferating endothelial cells, or endothelial cells exposed to turbulent flow (such as those at branch points of major vessels), may also express Cx43[28]. Generally, smooth muscle cells (and pericytes) of the medial layer are thought to express predominantly Cx43 and Cx45, but expression of Cx40 and Cx37 has also been detected in various vascular beds (and in-vitro models), suggesting expression of these proteins may be vessel, context, and species specific[29,30]. In the rat, arterial expression of Cx43 was found to vary between elastic and muscular arteries. Expression of Cx43 was detected in both the endothelial and medial layers of elastic arteries, but not in the media of muscular arteries[31]. Nevertheless, Cx43 is the connexin most commonly reported as being expressed in vascular smooth muscle cells (SMCs), especially in growing vessels, and although connexin 45 can certainly be detected, its function remains elusive[32].

II. Function-General Overview

The most conspicuous function of connexins expressed in the mature vascular wall is to support intercellular communication via the formation of gap junction channels. Studies exploring the coupling between various components of the vascular wall revealed that endothelial cells mostly express Cx40 or Cx37 and are chemically and electrically coupled[33-36]. Similarly, smooth muscle cells are [37]coupled to each other and are coupled

to endothelial cells as well. Coupling of smooth muscle cells to each other can be accomplished by forming Cx43 or Cx45 gap junctions, but coupling at the myoendothelial junction can vary (homotypic with endothelial Cx43 or heterotypic with endothelial Cx40 or Cx37) depending on the composition of the extracellular matrix (ECM), mechanical forces and/or proliferative state of endothelial cells[37,38]. Interestingly, it has been suggested that heterocellular coupling at the myoendothelial junction (in cases where both ECs and vascular smooth muscle cells (VSMCs) were found to express Cx37) can be differentially regulated such that Cx37 was excluded on both the endothelial and smooth muscle sides of the junction, allowing communication to be mediated by the other connexins expressed in each of those compartments[38]. This dual coupling of cells within and between the endothelial and smooth muscle layers supports reciprocal signaling between compartments of the vascular wall, allowing each of them to respond to local signaling events in a coordinated manner. Additionally, connexins engage in a number of protein-protein interactions with cytoplasmic proteins, which can also modulate vascular cell function.

In addition to supporting communication and proper function in the mature vessel, connexins (and gap junctions) also have a role in vascular development.

Because vascular connexins are sometimes co-expressed in the same cell type, it is tempting to speculate that their function is redundant and that loss of one connexin can be compensated by the presence (or up-regulation) of another. However, an overview of the existing literature reveals that such a view of connexin function is too simplistic[34]. Whether different connexin isoforms perform overlapping functions in a particular cell depends on the kind of cell and type of function being examined. Moreover, some studies

suggest that connexins are differentially co-regulated in various vascular tissues. For example, it has been reported that deletion of Cx40 results in a seemingly compensatory increase of both Cx37 and Cx43 in the medial layer of mouse aortas, but a reduction in Cx37 expression was observed in the endothelium[33]. Moreover, deletion of specific connexins from the genome, in ubiquitous or inducible manner, results in different vascular phenotypes. Taken together existing data suggest that vascular connexin expression is co-regulated in a cell or tissue specific manner, presumably reflecting need for unique inter and intracellular signaling states. Therefore, when exploring connexin function in the vasculature, it is important to consider their role in vascular development and homeostasis, while keeping in mind both channel dependent and channel independent mechanisms that connexins can support.

III. Function: Vascular Consequences of Connexin Deletion

As previously discussed, mice deficient in Cx40 survive to adulthood but are hypertensive due to dysregulated upstream conduction of vasodialatory signals and overproduction of renin[35,39,40]. Recently, it was also shown that Cx40 and Cx37 interact with endothelial nitric oxide synthase (eNOS) and loss of Cx40-/- (with concurrent reduction of Cx37) leads to reduced expression of eNOS and impaired vasodilation[41]. Mice deficient in Cx37 also survive to adulthood but females are infertile due to failure in ovarian cell signaling. These mice do not display obvious defects in arterial development or function. Cx37 deficient animals do, however, display absence of venous valves[42]. The abnormal arterial phenotypes associated with lack of either Cx40 or Cx37 are most consistent with a major role for these connexins in the adult endothelium, where the majority of vasodilatory signals originate. However, loss of both Cx40 and Cx37 together

results in embryonic death associated with vascular malformations and profound hemorrhage in multiple vascular beds[33]. Taken together these data suggest that functional overlap for Cx37 and Cx40 exists during development.

In contrast, deletion of Cx43 alone results in embryonic death due to abnormal development of RV outflow tract and abnormalities of the coronary vasculature[43]. Similarly, absence of Cx45 expression results in embryonic lethality due to inability to form arterial smooth muscle layer and heart failure associated with endocardial cushion defect[44,45]. Moreover, neither phenotype is consistent with abnormal endothelial cell differentiation (or vasculogenesis), suggesting that these connexins are critical for the development of the vascular wall but either their role in early endothelial function is limited or their loss can be well compensated for by other connexins.

Connexin 43 and Connexin 45 in Vascular Development

Connexins 43 and 45 can be detected in developing and mature blood vessels, but their individual contributions to vessel function remains incompletely understood. The embryonically lethal phenotype of either knockout suggests that these connexins are both critical to the development of the vasculature, but dissecting the exact mechanisms that each connexin isotype supports is challenging due the early stage of failure (in the case of Cx45) and the presence of both connexins in multiple systems. In order to fully capitalize on the clinical relevance of Connexins 45 and 43, with regard to vascular disease, their precise functions in the modulation of vascular cell growth, differentiation and homeostasis must be well understood. Recently, studies utilizing conditional or tissue specific knockout models have circumvented early embryonic lethality and multiple

system involvement[32]. Similar experiments utilizing reporter genes (such as LacZ) have been utilized to more precisely define the expression profile of these connexins throughout development. Moreover, studies focused on the exact mechanisms of embryonic failure in mice lacking Cx43 or Cx45 have shed light on the contribution of each to the development of distinct vascular beds and have uncovered similarities and differences. Some of the experimental outcomes and the insights gained into the developmental function of these connexins are discussed below.

I. Expression Overlap and Specificity in Arterial Tissues

Although expression of both Cx43 and Cx45 has been demonstrated in the smooth muscle layer of adult murine arteries, their presence in arterial endothelium is limited under homeostatic conditions. It is worth noting, however, that developmental and disease processes may alter this expression pattern. For example, conditions of mechanical stimulation and hemodynamic stress (often found at the bifurcation points of major vessels) have been reported to alter the expression of Cx43 in mature arterial endothelium[28,46]. Thus, when investigating the "normal" expression and function of Cx43 or Cx45 in vascular tissue, it is important to consider the implications of any ongoing developmental and adaptive processes. Expression of Cx45 is widespread and robust during embryonic development[44]. Although studies aimed at defining its precise expression pattern in the vasculature have suffered from technical challenges, it has been recently reported that Cx45 is expressed largely in the smooth muscle of mesenteric arteries, large conducting arteries, small arteries, and arterioles of the microcirculation[32]. Occasionally, Cx45 has been detected in the endothelial layer[44].

II. Vascular Consequences of Connexin 45 Deletion

Cx45 expression is widespread in the developing embryo, but the early failure (between E9.5 and E10.5) of Cx45 knockout embryos is attributed mainly to defects in vascular development[44], although heart development is also affected[45] Cx45 KO embryos exhibit seemingly normal vasculogenesis (differentiation of endothelial cells and endothelial cell tube formation) but subsequent acquisition and/or differentiation of mural cells is not observed in the embryo proper or the yolk sac[44]. Mural cells stabilize vessels and, upon forming a functional vessel wall, allow for regulated re-distribution of blood flow to various vascular beds. (For review of embryonic vascular development see: [47]) Consequently, Cx45 knockout embryos appear pale and anemic, exhibit delay in development at day E9.5 and fail by E10.5[44]. These observations are consistent with a role for Cx45 in formation of the vascular wall. However, deletion of Cx45 specifically in VSMC accomplished with a Cre/LoxP system driven by a Nestin promoter resulted in no obvious vascular defects [32,48,49], suggesting that Cx45 is necessary for the commitment of MCs to a mural cell phenotype but is dispensable for mural cell function thereafter. Taken together these data suggest that Cx45 is critical only during development but its function in the adult is either limited or well compensated for (possibly by Cx43).

III. Cardiovascular Consequences of Connexin 43 Deletion

Like Cx45, Cx43 is also widely expressed throughout embryonic development and in the adult. However, deletion of Cx43 causes developmental failure much later and in a manner different from that observed in Cx45 deficient embryos. Mice lacking Cx43 die from obstruction of the pulmonary outflow tract due to formation of pouch-like structures at the infindibulum, a structure located at the base of the pulmonary outflow tract[43]. Upon

closer examination infindibular pouches were found to contain ectopic VSMC, fibroblast, and endothelial cells. These structures were also found to exhibit an increased activation of vascular endothelial growth factor (VEGF), TGFβ and other signaling pathways controlling angiogenesis and vasculogenesis[50]. In addition to the infindibular pouches, Cx43 deficient embryos display abnormal formation of the coronary vasculature[50,51]. Specifically, mice deficient in Cx43 exhibit incomplete SMC coverage of the coronary arteries, narrowing of the coronary artery mouth and stem, formation of only a single major coronary artery, and abnormal origin of the coronary arteries[51]. These observations have provided some insight into the role of Cx43 during development of the coronary vasculature, a process with a somewhat unique source of vascular precursors.

Two migratory cell types govern coronary vascular development: neural crest cells (NCCs) and proepicardial cells (PECs). NCCs migrate into the developing heart from the somites and regulate the patterning of the coronary vasculature, but whether they contribute significantly to the population of vascular precursors is still unclear. Chick embryos with ablation of somite-derived NCCs still formed coronary vessels, but these were spatially disordered and anomalous[52]. In contrast, PECs derived from a transient embryonic structure undergo epithelial to mesenchymal transition (EMT) and migrate into the cardiac tissue in order to form all the precursors for endothelial cells, smooth muscle cells, and vascular fibroblasts[53-55]. Loss of coupling in NCCs or PECs resulted in altered migration rate. NCC-specific downregulation of Cx43 resulted in a decreased NCC migration rate, whereas overexpression of Cx43 resulted in increased NCC migration rate[51,56]. Thus, in the in-vivo setting the correct Cx43 gene dosage seems critical for normal development. That loss of Cx43 targeted specifically to either NCCs or

PECs results in some of the phenotypes seen in animals with global Cx43 deletion suggests that both cell types might be affected in the global deletion model and perhaps both contribute to the observed pathology. However, it is currently unclear what specific mechanisms supported by Cx43 are disrupted and how.

TGF-β signaling

The transforming growth factor beta (TGF-β) superfamily consists of nearly thirty members and includes the three main TGF-β isoforms (TGF-β1, TGF-β2, TGF-β3), activins, inhibins, nodals, and the bone morphogenic proteins (BMPs). Members of this family mainly act in paracrine fashion, but cases of autocrine and even endocrine signaling have been documented. Additionally, there are also a number of intracellular modulators, which can augment or repress TGF -β signaling. With such a diverse array of signaling molecules and their ubiquitous expression, it is perhaps not surprising that findings about the role of TGF-β in developmental and pathological settings are frequently contradicting. It is increasingly recognized that both the role and signal transduction mechanisms of TGF-β are tissue and context specific. The following section provides only a general overview of TGF-β signaling.

I. Overview of TGF -β Signaling

Members of the TGF-β superfamily, characterized by a conserved number and spacing of C-terminal cysteine residues, are secreted in the extracellular space in inactive form and require proteolytic cleavage for activation and subsequent signaling. Upon activation the peptide binds a serine/tyrosine surface receptor, and initiates several intracellular signaling pathways. TGF-β signal transduction can be sub-divided into canonical and

non-canonical pathways. Canonical pathways were discovered early on and include Smad signaling, whereas non-canonical pathways were only recently discovered and include RhoA, Rac, MEKK 1, and others. Canonical pathways necessitate translocation of Smad proteins to the nucleus where in cooperation with transcription factors or co-repressors they alter the cell's gene expression profile. Smad binding partners confer additional specificity to the TGF-β signaling response and provide the diverse array of physiological functions despite the limited number of canonical (Smad) mediators.

a) Synthesis and secretion:

Most members of the TGF-β family are dimeric secreted polypeptides. A conserved three-dimensional fold and a conserved number and spacing of cysteine residues at the C-terminus of the mature peptide characterizes the group[57]. The three mammalian isoforms (TGF-β 1, 2, and 3) have been found to function in autocrine, paracrine, and even endocrine manner[58]. However, most members of the family function in a paracrine manner. All of the TGF-β family members are secreted as longer propeptides and are proteolytically modified. Initially, they exist as pre-pro-TGF-β but the signal peptide is cleaved during transit in the rough endoplasmic reticulum (ER). Another cleavage occurs upon dimerization. The result is a combination of mature peptide and LAP (latent associated peptide) within secretory vesicles or extracellular space. LAP and the mature peptide remain together (non-covalently bound) to form the small latent complex (SLC). LAP keeps TGF-β in latent form by masking the receptor binding sites. It is possible for the LAP of SLC to covalently interact with large latent TGF-β binding protein (LTBP) in order to form LLC (large latent complex). By interaction with components of the extracellular matrix (ECM), LLC is able to confine TGB-β to particular locations.

Fibronectin and fibrillin are thought to be the most common LLC and LTBP interaction partners.

b) *Activation*

Although a wide variety physical/chemical changes can activate TGF-β, activation is most commonly achieved through enzymatic cleavage of the latent complex. A number of enzymes are capable of liberating TGF-β from the confinement of its chaperones and the ECM. Elastase, which is capable of cleaving fibrillin can result in liberation of the complex, enabling its diffusion away from the sites of secretion. BMP1/Tolloid family of proteases have been demonstrated to cleave the LTBP and liberate TGF-β, as have matrix metalloproteases (MMPs)[57]. Thus, activation of TGF-β is typically determined by the microenvironment and presence/ activity of protease enzymes.

c) *Receptors*

Members of the TGF-β family bind mainly to type I or type II receptors. There are seven known type I receptors and five known type II receptors (Type I receptors are also called activin receptor-like kinase (ALK)). Both receptor types are composed of an extracellular portion, single pass transmembrane portion, and intracellular portion. The cytoplasmic portions have serine/threonine kinase activity. Ligand binding links the constitutively active type II receptor to the dormant type I receptor. The type II receptor phosphorylates the type I receptor at the GS region (a serine/threonine rich region characterized by a TTSGSGSG sequence), activating its kinase activity[57,59]. In the absence of ligand, two proteins FKBP 12 and FKBP 12.6 prevent access to the GS region of type I receptor by directly masking it and maintaining a type I receptor conformation unfavorable for interaction. Phosphorylation of the type I receptor turns the GS region from a FKBP

binding site to Smad 2/3 binding site[60]. Type III receptors are also present on some cells. Type III receptors are also called auxiliary receptors (or accessory receptors) because they aid in binding and activation of type I and II receptors. Examples of type III receptors include betaglycan and endoglin (CD-105). Upon ligand binding, two type II receptors combine with two type I receptors to form a heterotetramer. After ligand binding the heterotetramer can be internalized via clatrin coated pits into early endosomes. From here, receptors can still interact with Smad protein and continue signal transduction. The seven type I receptors have been designated ALK 1-7. The five type II receptors are named ActRIIA, ActRIIB, BMPRII, TGF-BRII, and AMHRII. Type I receptors for the BMPs (bone morphogenic proteins) include ALK3, ALK6, ALK2, and ALK1. Type II receptors for the BMPs include BMPRII, ActRIIA, and ActRIIB[61]. Although different members of the TGFβ family utilize different Type I receptors, the TβRII is uniquely required for signaling and has become the most accepted way of abolishing TGF-β signaling in experimental models.

d) Signal Transduction: Canonical VS Non-Canonical Pathways

Canonical Smad-dependent pathway: Smads are a group of eight cytoplasmic proteins (Smad 1-8) responsible for continuing the signal after receptor activation. Smad proteins are divided into three groups: receptor activated (R-Smads), common mediator (Co-Smads), and inhibitory (I-Smads). R-Smads are phosphorylated by the type I receptor at an SSxS motif near their C-terminus. Smads 1,2,3,5, and 8 are designated as R-Smads. Smads 2 and 3 tend to be phosphorylated in response to TGF-β and activin signals, whereas Smads 1,5, and 8 tend to be phosphorylated in response to bone morphogenic proteins (BMPs). However, crossover in activation is possible as it was found that TGF-β

may also lead to activation of Smads 1 and 5[58]. Smad 4, the only Co-Smad found in mammals, can be activated by both TGF-β/activin and BMP signals. Smad 6 and Smad 7 are designated as I-Smads and are activated by BMP and TGF-β/activins respectively. These proteins are approximately 500 amino acids in length, and consist of two globular domains coupled by a linker region[60]. Smad proteins have two conserved domains named MH1 and MH2. (I-SMADs lack MH1 domain) The MH1 is also known as the N-terminal homology domain and MH2 is known as C-terminal homology domain. MH1 and MH2 are joined by a poorly conserved linker domain. The MH1 domain has been implicated in cytoplasmic anchoring, nuclear import, DNA binding, and transcriptional regulation. The MH2 domain has been implicated in Smad oligomerization and target gene transcription. Type I receptor activation results in phosphorylation of R-Smads (Smad 2 and 3 are given as the typical example, but it depends on the activating signal.) near their C termini. However, other proteins interact with R-Smads before activation occurs. SARA (Smad anchor for receptor activation), a membrane bound protein, is known to associate with non- phosphorylated R-Smads and aid in interaction with the receptor via its 80 amino acid Smad binding domain (SBD). Because SARA has higher affinity for monomeric Smads, it dissociates when R-Smads bind Smad4. It is helpful to note that SARA tends to interact with Smads 2 and 3 whereas its homolog, endofin, performs a similar task for the BMP pathway (Smads 1,5,8)[57]. In addition to SARA, hepatocyte growth factor-regulated tyrosine kinase (Hrs/Hgs) may also bind to Smads and aid in their interaction with activated receptors (its effect is synergistic with SARA). Both SARA and (Hrs/Hgs) contain a lipid binding FYVE domain. Dab2 (Disabled-2) is known to associate with active type I or II receptors and bridge them with Smad 2/3. In addition, TGF-β receptor

associated protein (TRAP I) and embryonic liver fodrin (ELF) have also been implicated in activation of R-Smads by active receptors. This list of supportive proteins is by no means exhaustive. There are many more proteins found to interact with Smads in the cytoplasm.

Upon phosphorylation of the R-Smad C-terminal SXS motif by the activated receptor, R-Smads couple with Smad 4 and translocate to the nucleus. Based on the findings of Chacko et al. in 2004[62] , it is believed that Smad 2/Smad 4 and Smad 3/ Smad 4 complexes exist as heterotrimers composed of two R-Smads and one Smad 4. (The study did not explore R-Smads other than Smad 2 and 3 but suggested that the ratio of 2:1 is preserved in Smad 1, which is BMP specific.) However, it is possible that a cofactor replaces one of the trimer subunits and leads to the formation of a heterodimer between R-Smads and Smad 4[62]. The exact composition of R-Smad oligomers seems to be a matter of some debate. It appears that the exact composition depends on interaction with specific cofactors, as affinities of Smads for each other seem to change as a result of cofactor interactions[60].

The selection of binding partners in the nucleus confers specificity to the TGF-β pathway. First, pathway specificity can be established at this stage. Some binding partners prefer the BMP specific Smads, whereas others prefer the TGF-β/activin activated Smads. Second, cell type specificity can be established at this stage as some cells may lack the Smad binding partners necessary to repress or activate a gene. Third, target gene specificity (and specific transcriptional effect specificity) can be accomplished as Smad/binding partner complexes need specific target promoters (or repressors) to be in place[60]. Interactions of Smad proteins with their respective binding

partners ultimately leads to the activation or repression of target genes, and thus alteration in cellular behavior or morphology.

Non-Canonical Smad independent pathways include Rho, ROCK, MAPK, JNK and are much less well characterized. Although it was initially thought that these factors acted mostly on actin polymerization and fiber formation, there is increasing recognition that some can have effects on transcription and may be involved in vascular development. For example, Rho GTPase family member RhoA has been shown to be involved in the regulation of TGF-β signaling in smooth muscle cells. Specifically, dominant negative and constitutively active forms of RhoA were found to inhibit or augment (respectively) phosphorylation and nuclear translocation of Smad2/3. In addition, it has been found that Rho A action depends on PKN and p38 MAP kinase[63].

II. TGF-β in Vascular Development

That TGF-β is critical for proper vascular development is clear from the numerous studies ablating various components of TGF-β signaling. Although difficult to interpret with regard to tissue-specific vascular function, these initial reports collectively highlighted two general points: First, while some of the TGF-β signaling components can be well compensated for, others cannot. Second, ablation of TGF-β signaling components does not always produce identical phenotypes in all vascular beds, suggesting that vascular cells of different origin utilize somewhat different signaling cascades during vessel formation and maturation. Better understanding of TGF-β signaling and recent advances in tissue specific deletion, however, have allowed for a better evaluation of

TGF-β's contribution to the processes of vasculogenesis, angiogenesis, and vessel maturation.

A generic deletion of the gene encoding TGF-β1 results in 50% of the embryos failing at E10.5 due to severe vascular and hematopoietic system defects[64]. The remaining 50% survive, likely due to signaling redundancy or transplacental transfer of TGF-β, but die within three weeks of birth due to multifocal inflammatory disorder[65,66]. Because both hematopoiesis and vasculogenesis were affected (and because both processes originate from the same cluster structures), these data suggested that TGF-β signaling is necessary at a very early point in blood vessel formation, perhaps as early as endothelial cell differentiation or A-V specification. Upon differentiation and endothelial tube formation, endothelial cells undergo ALK1 dependent arterio-venous (A-V) fate specification. In support of this, it was found that deletion of ALK1, a type I TGF-β receptor that is present and active in the endothelium, leads to disrupted A-V specification in the endothelium marked by the presence of numerous A-V shunts and reduced expression of Efnb2, an artery-specific endothelial marker[67]. This phenotype was somewhat reminiscent of human patients afflicted with hereditary hemorrhagic telangiectasia (HTT), a genetic disorder presenting with abnormal blood vessel formation in multiple tissues. Interestingly, in addition to the A-V specification defects, embryos deficient in ALK1 also displayed a complete absence of vascular smooth muscle cells.

In addition to vasculogenesis and endothelial A-V fate specification, TGF-β signaling is also necessary at subsequent stages of blood vessel formation, namely mural cell differentiation. A role for TGF-β in smooth muscle cell differentiation was readily deduced from the apparent loss of smooth muscle cells in embryos with disrupted TGF-β

signaling but initially the proposed underlying mechanisms were highly contentious due to the underlying complexity and the limitations of non tissue-restricted knock out models. There are two common strategies for accomplishing tissue-specific deletion. One strategy is to ablate a component of the TGF-β signaling axis that is expressed (or active) only in a particular tissue (ALK1 for example, which is mostly active in the endothelium). A second strategy for tissue-specific ablation of TGF-β signaling utilizes simultaneous introduction of a "floxed" gene encoding a critical mediator (TβRII receptor, for example) and Cre recombinase gene under regulation of a cell type-specific gene promoter (smooth muscle 22 alpha, for example). Although there are limitations to both systems, these experimental designs have contributed significantly to our understanding of TGF-β's role in vascular development.

Points of Intersection: Connexin Dependent, TGF-β Mediated Signaling in Cardiovascular Development

Because deletion of Connexin 43 (or Cx45) or disruption of TGF-β signaling display vascular development detriments with some overlapping features, it is tempting to speculate that there is a direct mechanistic link between the two. Although such conclusion based only on the existing in-vivo data might be premature, this interpretation is strengthened by the existence of several in-vitro models of EC-SMC interaction that clearly demonstrate necessity for Cx43 or Cx45 in EC-induced, TGF-β dependent SMC phenotype induction and maintenance. A graphical summary of the signaling components involved in recruitment and differentiation of mural cells is provided in Figure 2.

These approaches relied on genetic ablation, siRNA-mediated silencing, or pharmacological blockade of Cx43. It was consistently observed that Cx43 (or Cx45) is necessary for endothelial cell-induced differentiation of mesenchymal cells toward a mural cell phenotype (and in a similar manner for the maintenance of a differentiated phenotype in cultured smooth muscle cells). However, whether communication or protein-protein interactions (or both) are truly the basis of Cx43-mediated action remains somewhat difficult to interpret as loss of Cx43 expression disrupts both parameters and most of the pharmacological agents available have been shown to have non-specific effects. There are, however, at least some indications that both communication and protein-protein interactions are both involved in endothelial cell-induced mural cell differentiation.

I. Communication Dependent Events

Although it has been the accepted view that Cx43- (or Cx45-) mediated communication is necessary for EC-induced (TGF-β dependent) upregulation of smooth muscle specific proteins in mesenchymal (MC) or de-differentiated smooth muscle cells, the identity of the communicated signal remains uncertain. This communicated signal must be 1) synthesized (and/or transiently activated) in ECs or MCs only, 2) small enough to traverse gap junction channels, and 3) able to induce activation of TGF-β in the extracellular space. It has been suggested that serotonin is that critical messenger in the setting of pulmonary artery endothelial and smooth muscle cells[68,69]. Whether or not this is the case with mesenchymal cells and endothelial cells in the developmental setting (or in different vascular beds) remains to be determined. Herein we explore the possibility that calcium might be the signal transmitted between ECs and MCs.

In addition to the nature of the communicated signal, it is also unclear whether communication persists after the point of TGF-β activation or whether it is modified during the differentiation process. If so, how is the modification achieved, and is it absolutely necessary for differentiation to proceed? Stated differently, is communication alone sufficient for EC-induced differentiation? Could another vascular connexin replace Cx43 or Cx45 in their apparently unique ability to support EC-induced differentiation? These points are addressed in the following chapters.

Due to the ubiquitous nature of TGF-β signaling and wide array of cell types utilizing Cx43 to support GJIC, it is perhaps not surprising that reports describing the effect of TGF-β treatment on Cx43 expression and function are highly variable and often conflicting. Some studies have reported that TGF-β down-regulates Cx43 expression and reduces GJIC[70,71] with a concurrent change in PKC-target site phosphorylation, whereas others reported no changes in Cx43 expression and reduced phosphorylation[72]. Therefore, as with other aspects of TGF-β signaling, the effect on GJIC, Cx43 expression and phosphorylation should be considered in the cell specific context. Nevertheless, most studies suggest that TGF-β treatment results in decreased GJIC.

II. Protein-Protein Interactions

Although there are numerous reports of altered Cx expression in response to TGF-β signaling, reports of direct interaction between gap junction proteins with members of TGF-β signaling pathways are few. Using a cell culture model, Dai et al reported that Cx43 competes with Smad2/3 for tubulin binding sites[73]. The study demonstrated that Cx43 binds to tubulin and increases the number of Smad2/3 molecules available for phosphorylation, thereby modulating the sensitivity of TGF-β signaling. Although this

study was performed in cardiomyocytes, it is likely that this mechanism of TGF-β signaling modulation is conserved across multiple cell types including mural precursors. Therefore, it is possible that in addition to supporting EC-induced activation of TGF-β, Cx43 participates in signaling events downstream of the TGF-β receptor.

Background Summary:

In recent years strategies for manipulation of Cx43 expression and function have proven of immense therapeutic value for the treatment of chronic dermal wounds[74,75] and corneal injuries[76,77]. The success of these strategies has highlighted the potential value that Cx biology holds for the purposes of therapeutic intervention in various disease states. Because Cx proteins are critically involved in vascular tissue development and homeostasis, it is plausible that the role of Cxs in the vasculature can be similarly exploited for the purposes therapeutic intervention in vascular disease. However, understanding the underlying mechanisms is critical for developing successful therapeutic strategies.

The Background section has provided an overview of blood vessel formation, the role of connexins in the arterial development (and homeostasis), and an overview of TGF-β signaling. The studies reviewed herein clearly demonstrate a role for Cx43 (and Cx45) in cardiovascular development as evidenced by the failure of embryos to survive in the absence of either of these proteins. However, in-vivo studies utilizing Cx deletion provide only limited mechanistic insight due to the involvement of multiple organ systems and compensatory mechanisms. Thus, utilization of in-vitro models supplementing in-vivo work in order to dissect the intricate vascular development mechanisms on the cellular

and molecular levels is highly desirable. The in-vitro studies described in the background section support a role for Cx43 or Cx45 in EC-induced differentiation of MCs toward a mural cell phenotype that is TGF-β dependent. Specifically, these studies suggest that expression of Cx43 or Cx45 in MCs is permissive of an interaction between ECs and MCs that results in activation of TGF-β in the extracellular space and differentiation of MCs toward a mural cell phenotype. However, little is known about what aspects of the connexin structure and function are truly necessary and sufficient to support the differentiation process. For example, the necessity and sufficiency of the channel and the carboxyl terminus (a major regulatory and protein-protein interaction domain) have never been independently assessed. Further, it is unknown what messengers (if any) are communicated between ECs and MCs during blood vessel formation and whether the communication between ECs and MCs is regulated. Providing answers to some of these questions is critical for elucidation of the signaling pathways between ECs and MCs that can eventually be targeted to augment or attenuate vascular growth. The chapter titled "THE PRESENT STUDY" presents the specific aims designed to address some of these questions.

Figure Legend 1: General Overview of Blood Vessel Formation- Schematic Representation

Blood vessel formation begins with the generation of angioblasts from multipotent embryonic cells. Angioblasts congregate to form cluster-like structures termed “blood islands”. The cells of the outer layer eventually differentiate into endothelial cells (red circular symbols), whereas cells of the inner mass differentiate to form the cellular constituents of blood. Blood islands fuse and form a network of endothelial cell tubes termed “the primary capillary plexus”. Endothelial cells secrete chemoattractants and mitogens that recruit mural progenitor cells (shown in orange) from mesenchymal tissue. Upon contact, and gap junction formation, endothelilal cells induce differentiation of mesenchymal cells toward a mural cell (smooth muscle cell or pericyte) phenotype. Further remodeling of the initial plexus occurs mainly through the process of angiogenesis and results in the formation of a circulatory system that contains the venous (blue) and arterial (red) components connected by capilliary beds (white). Throughout development and in the adult pathological stimuli may induce a state of insufficient or excessive vascular formation.

FIGURE 1

General Overview of Blood Vessel Formation- Schematic Representation

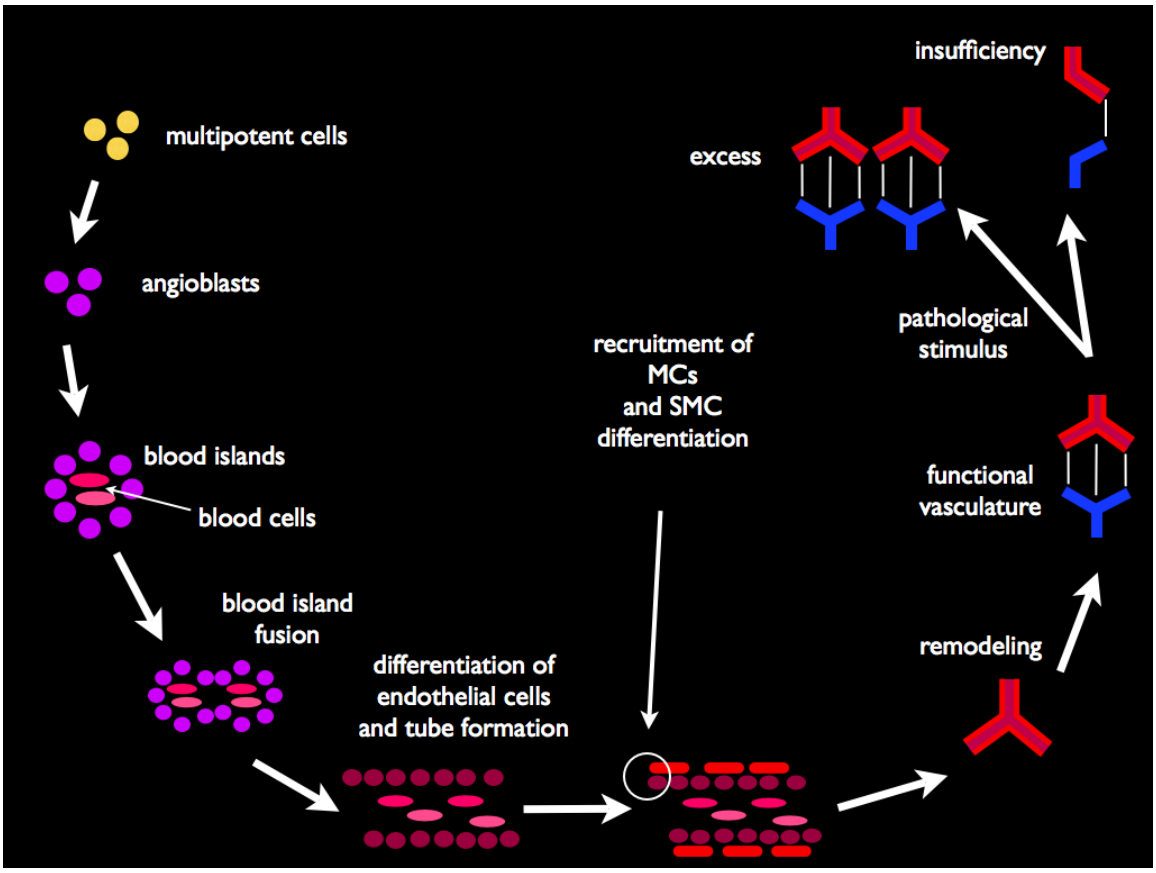
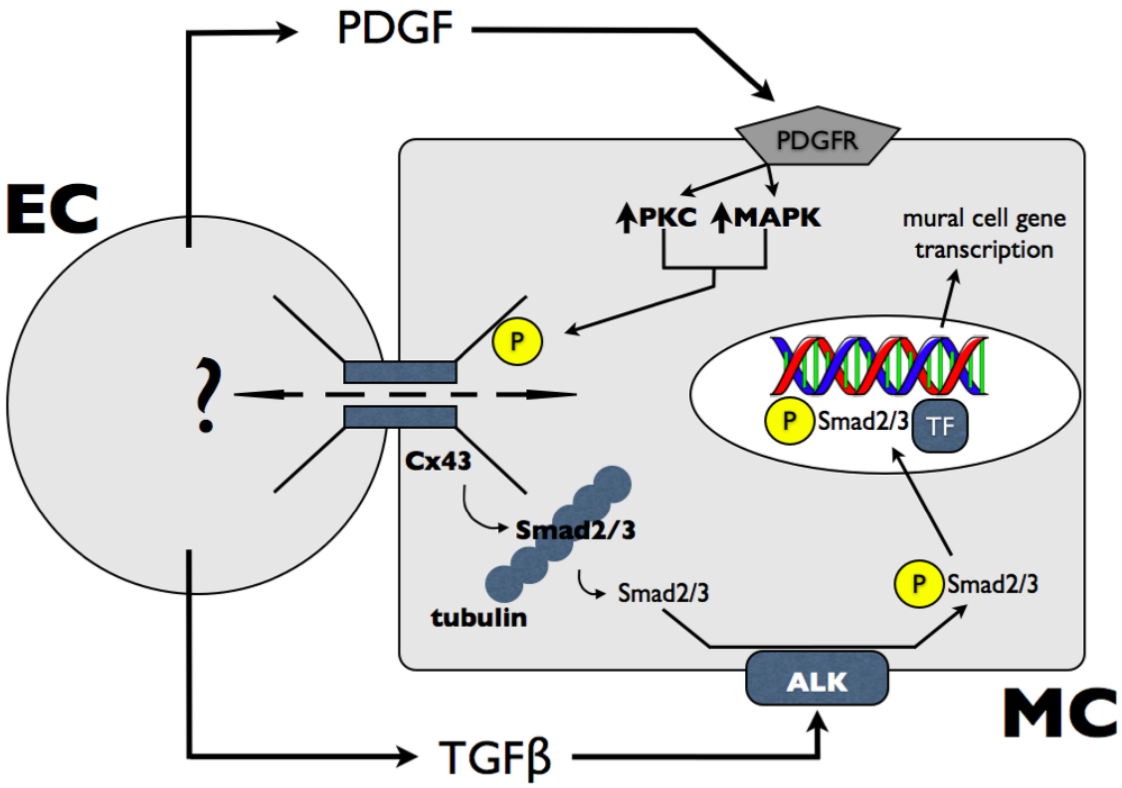


Figure Legend 2: Summary of Hypothesized Signaling Events During Endothelial Cell- Induced Mesenchymal Cell Differentiation Toward a Mural Cell Phenotype

Endothelial cells (ECs) secrete platelet derived growth factor (PDGF), which acts as a chemoattractant and mitogen for mesenchymal cells (MCs). The activation of protein kinase C (PKC) or mitogen activated protein kinase (MAPK) pathways is hypothesized based on reports from other contexts of PDGF receptor stimulation. Mesenchymal cells migrate toward and ultimately contact endothelial cells. Upon contact, heterocellular gap junctions are formed. This sequence of events results in TGF-β activation in the extracellular space, which is secreted in inactive form by both ECs and MCs. TGF-β binds type 2 (TβRII) receptors, which recruit and activate Type I (Aktivin-Like Kinase (ALK)) receptors. Active ALK receptors recruit and phosphorylate Smad 2/3 molecules that translocate to the nucleus, bind additional transcription factors (TFs) and induce the expression of mural cell genes. It should be noted that in addition to supporting EC-MC intercellular communication, which is necessary for activation of TGF-β, Cx43 can compete with Smad2/3 for tubulin binding sites, thereby making Smad 2/3 available for phosphorylation and augmenting TGF-β signaling.

FIGURE 2

Summary of Hypothesized Signaling Events During Endothelial Cell - induced Mesenchymal Cell Differentiation Toward a Mural Cell Phenotype



CHAPTER 2: THE PRESENT STUDY

The broad goal of this study is to further clarify the role of connexins, specifically Cx43, in endothelial cell-induced differentiation of mesenchymal cells toward a mural cell phenotype. The methods, results and conclusions of for this study are presented as a series of three manuscripts prepared for publication in collaboration with several colleagues. The prepared manuscripts are appended to this dissertation in appendices A, B, and C, which follow this section. The specific aims for the combined body of work are as follows:

Specific Aim 1a: Determine whether a functional channel is necessary for EC-induced mural cell differentiation.

Specific Aim 1b: Determine whether the carboxyl terminus (a major regulatory and protein binding domain) of Cx43 is necessary for EC-induced, mural cell differentiation.

Specific Aim 2a: Determine whether MAPK and PKC target sites within the carboxyl terminus of Cx43 are necessary for EC-induced mural cell differentiation.

Specific Aim 2b: Determine whether Cx40 (which is also found in the vasculature but is not directly targeted by MAPK) is capable of supporting mural cell differentiation.

Specific Aim 3: Determine whether calcium elevation in ECs is sufficient to replace Cx43-mediated gap junction formation, leading to TGF-β activation and mural cell differentiation.

The manuscripts pertaining to each of these specific aims were included in appendices A, B, and C (for aims 1, 2, and 3, respectively). A brief summary of the experimental outcomes for each specific aim follows.

Specific Aim 1: A) Determine whether a functional channel is necessary for EC-induced mural cell differentiation. B) Determine whether the carboxyl terminus (a major regulatory and protein-binding domain) of Cx43 is necessary for EC-induced, mural cell differentiation.

This aim was investigated in the study included in APPENDIX A. Prior to completion of this work, it was the prevailing view that Cx43 (or Cx45) channel function is necessary for the communication of a critical message between ECs and MCs that results in TGF-β activation and mural cell differentiation. However, the need for channel function had never been directly addressed. Previous experiments relied on either connexin gene knockdown/ knockout strategies or on pharmacological blockers of Cx43. However, knockdown and knockout strategies interfere with both communication-dependent and communication- independent signaling events, while pharmacological gap junction blockers suffer from lack of specificity. Moreover, it was unknown whether communication alone, in the absence of the carboxyl terminus (CT, a major regulatory and protein binding domain) is sufficient to support the process. If so, it would be expected that connexin specificity would not be critical for EC-induced differentiation

and any connexin capable of supporting GJIC between ECs and MCs would be able to support this process. The approach used in this study was able to circumvent the limitations of previous studies by relying on re-introduction of channel dead or truncated forms of Cx43 into mesenchymal cells lacking Cx43 expression. The major finding of this study was that both channel functionality and presence of the CT domain are necessary for EC-induced mural cell differentiation and that neither alone is sufficient to support the process.

Specific Aim 2: A) Determine whether MAPK and PKC target sites within the carboxyl terminus of Cx43 are necessary for EC-induced mural cell differentiation. B) Determine whether Cx40 (which is also found in the vasculature but is not directly targeted by MAPK) is capable of supporting mural cell differentiation.

This aim was investigated in a collaborative effort with Dr. Jennifer Fang and Dr. Karen Hirschi (APPENDIX B). That the CT of Cx43 is necessary for EC-induced differentiation strongly suggests that unique regulatory events (or binding interactions) confer Cx specificity to the differentiation process. However, it was unknown whether Cx isoforms other then Cx43 and Cx45 might be capable of supporting mural cell differentiation. Moreover, it was unclear which (if any) specific regulatory sites were involved. We used the same experimental paradigm as Specific Aim 1 and introduced constructs of Cx40 or constructs of Cx43 with serine to alanine substitutions at the MAPK target sites (S255,279, and 282) or the PKC target site (S368). There were two major findings of this study. First, we found that the Cx43 MAPK target sites were necessary, while the PKC site was dispensable, for EC-induced mural cell differentiation.

Second, we found that unlike Cx43, Cx40 expression in the MCs is not capable of supporting EC-induced differentiation, despite forming functional gap junctions between ECs and MCs. These data reaffirm the notion that in addition to GJIC, other aspects of Cx function are also critical to the differentiation process.

Specific Aim 3: Determine whether calcium elevation in ECs is sufficient to replace Cx43-mediated gap junction formation and lead to TGF-β activation and mural cell differentiation.

This aim was investigated in a collaborative effort with Dr. David Kurjiaka, Dr. Karen Hirschi, and Dr. Scott Boitano (APPENDIX C). In order to address this aim, we used the same EC and Cx43 deficient MC types as the ones used in the previous two aims. We hypothesized that elevation in EC intracellular calcium with ionomycin will lead to activation of TGF-β in the extracellular space and mural cell differentiation in naïve MCs exposed to the medium conditioned by ionomycin treated ECs. The outcome of this study was intriguing. Although we ultimately concluded that an increase in EC intracellular calcium concentration was not likely to cause TGF-β activation and subsequent mural cell differentiation, the data suggested that calcium elevation might be a trigger for a redundant pathway that is capable of inducing an upregulation of smooth muscle alpha actin (an early marker of differentiation) via a related signaling pathway.

Summary:

Taken together, these studies support a role for connexins in EC-induced mural cell differentiation that goes beyond that of a simple conduit connecting the cytoplasmic compartments of neighboring cells. Although additional studies will be necessary to

describe the exact mechanisms, the data presented herein suggest that connexin-specific structural and functional features (communicating pore and regulatory domain) contribute to the process. These studies provide new directions for continued investigations into connexin dependent mechanisms relevant to vascular development and regeneration.

CHAPTER 3: SUMMARY AND FUTURE DIRECTIONS

As discussed in CHAPTER 2, prior to the completion of this work several studies in the published literature supported the notion that communication via Cx43 gap junctions is necessary for EC-induced differentiation of mesenchymal cells toward a mural cell phenotype, but this conclusion was reached by using experimental paradigms that were not optimally suited to test this hypothesis. Moreover, whether the CT domain of Cx43 was critically involved was also unknown. The data presented herein support the hypothesis that channel functionality and presence of the carboxyl terminal domain of Cx43 are both necessary, and neither alone is sufficient, for endothelial cell-induced mesenchymal cell differentiation toward a mural cell phenotype. Unlike Cx43 wild-type expressing MCs, MCs expressing channel dead or truncated versions of Cx43 did not undergo EC-induced mural cell differentiation. MCs expressing mutant forms of Cx43 were, however, found to increase smooth muscle alpha actin and calponin expression in response to stimulation with exogenous TGF-β, but this increase appeared to be somewhat attenuated relative to the Cx43 wild-type expressing MCs. Thus, it is not currently clear whether the inability of MCs to differentiate in response to co-culture with ECs is entirely due to inability to support signaling interactions with ECs that are necessary for TGF-β activation, or whether failure to differentiate is in part due to inefficient TGF-β signaling.

One possibility is that changing the conformation of Cx43 to compromise channel function, truncating its carboxyl terminal domain, or otherwise modifying this protein leads to inability to efficiently activate downstream mediators of TGF-β signaling. To test this hypothesis, the ability of MCs expressing wt or mutant Cx43 to activate downstream mediators of TGF-β signaling could be compared. Upon exposure to varying concentrations of TGF-β, activation of both canonical (Smad mediated) and non-canonical (MAPK, RhoA, etc) TGF-β signaling pathways could be assessed in parallel. This way one could determine whether the pathways are comparably activated or whether alternative downstream signaling pathways might be favored in MCs expressing mutant forms of Cx43.

Alternatively, it is possible that the TGF-β signaling pathway in the channel dead and truncated Cx43 expressing MCs is sufficiently intact (and comparable), but modified versions of Cx43 are not capable of supporting EC-induced activation of TGF-β. To test this hypothesis, levels of activated TGF-β in the extracellular space could be monitored upon co-culture of ECs with Cx43 wt or mutant expressing MCs.

Lastly, it is important to note that the two scenarios described above are not mutually exclusive. It is possible that both inability to support EC-MC signaling events and altered TGF-β signaling both contribute to the failure of MCs expressing channel dead or truncated Cx43 to undergo EC-induced mural cell differentiation.

The data presented herein also supported the hypothesis that MAPK targeted serines at positions 255,279 and 282 in the CT domain of Cx43 are critical, and PKC targeted serine at position 368 is dispensable, for EC-induced differentiation. This observation implicates MAPK signaling in the differentiation process, but the exact MAPK isoforms

involved, their activating mechanisms, and the functional consequences of Cx43 phosphorylation are not known. Thus, further studies will be necessary to describe the molecular details of these signaling interactions. Isoform specific inhibitors for ERK, p38, and other MAPK isoforms are becoming commercially available and their use in EC-induced mural cell differentiation models can, at least in principle, be used to determine which MAPK isoform is involved in EC-induced differentiation. If the identity of the MAPK isoform is determined, then the rest of the signaling pathway could be deduced and investigated further.

The hypothesis that Cx40, like Cx43 and Cx45, is able to support EC-induced mural cell differentiation was not supported. Interestingly, Cx40 expressing MCs did not differentiate in co-culture with ECs, despite being able to support gap junction intercellular communication with ECs, suggesting that specific aspects of Cx function are involved. Whether the unique ability of Cx43 or Cx45 to support EC-induced mural cell differentiation stems from the ability to regulate the channel to a specific permeable (and/or subconductive) state or from the ability to participate in unique protein binding interactions (or both) could be investigated if the function of these two parameters are separated. One way to address these questions is to transfect Cx43-/- MCs with chimeric Cx constructs that allow for proper channel functionality and/or presence of the carboxyl terminal domain (for example Cx40 channel with Cx43 carboxyl terminus). Experiments utilizing chimeric protein constructs may yield additional information about whether (and how) communication is regulated during the differentiation process.

The hypothesis that calcium elevation in the ECs is sufficient to initiate TGF-β activation and subsequent mural cell differentiation was not entirely supported. Although medium

conditioned by ionomycin treated ECs was capable of increasing SMA expression in MCs, this response was mediated not by TGF-β, but likely a related ligand utilizing the same type I (ALK) receptors. Thus, elevation of EC intracellular calcium concentration (at least as induced by ionomycin) is unlikely to be the physiologically relevant mechanism for initiating activation of TGF-β during EC-induced mural cell differentiation. Nevertheless, calcium signaling may still be necessary for EC-induced mural cell differentiation. One way to test this hypothesis is with the use of calcium chelators such as BAPTA in in-vitro models of mural cell differentiation. If calcium signaling is indeed necessary for EC-induced mural cell differentiation, then differentiation should not be observed when ECs and MCs preloaded with a calcium chelator are co-cultured. These experiments are difficult to conduct using the current experimental setup because the period of time during which BAPTA treatment is effective in suppressing calcium signaling is far shorter then the time course of the differentiation experiments. In order to circumvent this experimental challenge modifying the current experimental setup will be necessary.

Lastly, it is important to examine the context specificity of the current mural cell differentiation model. During development, mesenchymal cells that form the blood vessel wall can be recruited from different germ layers depending on the vascular bed. Consequently, commitment toward mural cell lineage might occur in response to different sets of signaling molecules. Thus, further studies should be conducted to determine whether the necessity for gap junctional communication between ECs and different types of MCs is always necessary and whether the signaling cascades involved

in differentiation have common elements. If common elements can be identified, perhaps they can most effectively be targeted for the purposes of therapeutic interventions.

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APPENDIX A
(Prepared for Submission at *ATVB*)

CONNEXIN 43 CHANNEL FUNCTION AND CARBOXYL TERMINAL DOMAIN ARE NECESSARY, BUT NEITHER ALONE IS SUFFICIENT, FOR ENDOTHELIAL CELL-INDUCED MURAL CELL DIFFERENTIATION

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Abstract:
Objective- Endothelial cell (EC)-induced mesenchymal cell (MC) differentiation toward a mural cell phenotype is TGF-β mediated and requires heterocellular gap junction formation; connexin 43 (Cx43)-composed channels enable TGF-β activation and endothelial-induced mural cell differentiation. The functional features of Cx43 required in this context have not been elucidated. We hypothesized that gap junction intercellular communication (GJIC) and regulatory sites in the carboxyl terminus (CT) are both necessary to support this differentiation process.
Methods & Results - Cx43 deficient MC cells, infected or transfected with either wild-type Cx43 (Cx43wt), Cx43-T154A (channel dead), or Cx43tr (residues 258-382 deleted), were co-cultured with ECs and EC-induced mural cell differentiation assessed. Cx43wt expressing MCs were induced by ECs to upregulate the mural cell proteins smooth muscle alpha actin and calponin. Neither the channel-dead nor truncated forms of Cx43 supported EC-induced MC differentiation. However, MCs expressing channel dead or truncated Cx43 up-regulated mural cell protein expression when stimulated with exogenous TGF-β.
Conclusions-These data demonstrate that GJIC and the regulatory sites in the CT domain are both necessary, and neither alone is sufficient, for Cx43-supported EC-induced mural cell differentiation. Moreover, the data suggest that both functional features of Cx43 are necessary for EC-induced activation of TGF-β, and are not required for but may nevertheless modulate the downstream signaling events within MCs.

Key Words: endothelial cell, mesenchymal cell, mural cell differentiation, TGF-β

Introduction:

During the initial stages of blood vessel development angioblasts differentiate into endothelial cells (ECs) and form a highly branched network of tubes that serves as a blueprint for the developing vasculature.[1] The ECs secrete PDGF-B, which acts as a chemoattractant and mitogen for mural cell precursors in the mesenchymal tissue.[2-4] Upon contact with ECs, these mesenchymal cells (MCs) undergo TGF-β-dependent differentiation toward a mural cell phenotype and give rise to the smooth muscle cells (SMCs) and pericytes of the vascular wall, which depends on cell-cell interaction, gap junction channel formation and extracellular matrix deposition.[5-7] Mural cells stabilize nascent vessels and regulate blood flow by supporting coordinated vasoconstrictions/ dilations. Further branching and remodeling of the initial vascular network, driven by local imbalances in oxygen supply and demand, are mainly accomplished by angiogenesis and anastomosis of new capillaries with existing ones. This process is complex and requires crosstalk from multiple signaling pathways. (For perspectives see Holderfield et al, 2008.[8]) The end result is the formation of a highly organized, tightly regulated vascular system that supports the growth and development of other tissue types. While numerous in-vitro and in-vivo studies have demonstrated that connexin expression is critical for proper vascular development and homeostasis, it is not entirely clear what aspects of connexin structure and function are truly necessary and sufficient to support the process. Elucidation of these mechanisms is critical for developing therapeutic strategies for targeting diseases with vascular involvement.

Gap junctions are aggregates of intercellular channels that serve as ionic and molecular conduits by directly coupling the cytoplasmic compartments of adjacent cells. Each gap junction channel comprises six connexins (Cx), a family of structurally related transmembrane proteins.[9,10] Of the more than twenty connexin isoforms identified to date, only four (Cx43, Cx40, Cx45 and Cx37) are expressed in the vasculature where their precise expression varies among cell types, vascular beds and homeostatic/ proliferative state.[11-15] Endothelial cells of arteries most commonly express Cx37 and Cx40, whereas proliferating and capillary endothelial cells also express Cx43. Mural cells predominantly express Cx43, although Cx45 expression has also been documented.[14-16]

That connexins are necessary for vascular development and homeostasis is evident from the phenotypes that result from their ablation.[18-23] However, only ablation of Cx45 or Cx43 leads to either embryonic or early post-natal death with profound cardiovascular phenotype, suggesting that these connexins are indispensible for proper formation of the cardiovascular system (albeit at different developmental time points). Furthermore, that failure of Cx43 or Cx45 deficient animals occurs despite these proteins being co-expressed in the adult vascular wall suggests that they have only partially overlapping roles that cannot always be fully compensated by the remaining connexin, or that they are differentially expressed at critical times during embryonic development.

Studies utilizing in-vitro models of blood vessel development (and EC-induced contractile phenotype) have suggested two possible (and not mutually exclusive) mechanistic functions performed by connexins during blood vessel formation and maintenance. First, support of GJIC between EC and MCs enabling TGFβ activation.[7,24-26] Second, protein-protein interactions of connexins with signaling pathways involved in mural cell differentiation (and maintenance of a contractile phenotype).[27] Using an in-vitro assay we (and others) have previously reported that Cx43 (or Cx45) mediated GJIC is necessary for EC-induced mural cell differentiation.[3,7,24,26]

However, no studies have yet addressed whether the carboxyl terminus (CT) or a functioning pore domain are necessary to support EC-induced mural cell differentiation. This question is relevant because the CT domain is the least conserved region between the different connexin isoforms and confers to gap junction channels (at least in part) their unique molecular permeability, electrical conductance states, regulatory behavior, and the ability to interact with different binding partners.[27-30] (For an in-depth discussion of the structural basis of gap junction channel behavior see Ek Vitorin and Burt.[28]) In addition, it has been previously demonstrated that expression of the carboxyl terminal domain of Cx43 alone (in the absence of a functional channel) is sufficient to reduce cell growth and induce changes in cell morphology associated with regulation of the actin cytoskeleton.[31,32] Furthermore, previous studies testing the necessity of communication alone relied on knockout/knockdown strategies, which interfere with both communication dependent and communication independent events, or on gap junction channel blockers, the function of which is non-specific.[24,33] Herein we use a previously described EC-MC co-culture model[7] that relies on neither knockdown/knockout of existing Cx43 nor pharmacologic blockade of GJIC. Instead, we utilized MCs isolated from Cx43-/- embryos with introduced expression of wild-type, channel-dead or a truncated version of Cx43, and assessed their ability to undergo EC- and TGF-β induced mural cell differentiation. Our results suggest that Cx43 mediated communication is necessary, but in the absence of the carboxyl terminus, not sufficient to support EC-induced MC differentiation toward a mural cell phenotype.

Results:

We previously reported that $Cx43^{-/-}$ MCs fail to undergo EC-induced mural cell differentiation without re-introduction of Cx43 or increased expression of Cx45, suggesting that either Cx43 or Cx45 is necessary and sufficient for this process.[7,26] Connexins support growth and differentiation by one or more of three possible mechanisms, intercellular signaling through gap junction channels, transmembrane signaling through connexons, and intracellular signaling through protein-protein interactions. Which of these mechanism(s) is essential for Cx43-supported, EC-induced MC-differentiation remains unclear and represents the focus of this study.

Cx43wt, Cx43-T154A and Cx43tr expression and function in Cx43$^{-/-}$ MCs.
As previously reported, immunostaining of Cx43$^{-/-}$ MCs (MC43$^{-/-}$) revealed no detectable Cx43 signal (Fig. 1A) and NBD-m-TMA dye coupling studies re-confirmed that MC43$^{-/-}$ cells (Fig.1 B-E; n=10) were not coupled. In contrast, MC43$^{-/-}$ cells infected with Cx43wt (*L*MC43-wt) exhibited robust Cx43 expression and proper localization of the protein (Fig. 1F) to appositional membranes, signifying formation of gap junction plaques. As expected, consistent intercellular NBD-m-TMA dye transfer was observed in these cells (Fig. 1G-J; n=15).

Similarly, MC43$^{-/-}$ infected with Cx43-T154A (*L*MC43-T154A) displayed robust protein expression (Fig. 2A,D) and readily identifiable gap junction plaques (Fig. 2A-inset). As expected[34], these cells were not dye coupled (n=6; Fig. 2B,C). Cx43 expression in Cx43tr infected MC43$^{-/-}$ (*L*MC43-tr) could not be detected by Western or immunocytochemical techniques (Fig. 2D,E) as the antigenic sequence was absent. However, as expected from previous reports[35,36] on the behavior of Cx43tr channels, NBD-m-TMA dye-coupling in these (n=31; Fig 2F-I) and *L*MC43-wt was comparable, indicating the presence of functional Cx43tr junctions are formed by *L*MC43-tr cells.

*L*MC43-tr cells, like Re43 cells[7], also formed functional gap junctions with BAECs. Fig. 2J-K shows co-cultures of these cells wherein the ECs were prelabeled with PKH26. In this example the NBD-m-TMA dye was injected into an EC (arrow in Fig. 2J; PKH26 labeling in 2K) and appearance of NBD-m-TMA in neighboring cells imaged after one minute (Fig. 2L). Dye was readily detected in an adjacent *L*MC43-tr cell (arrow head, Fig. 2L) as well as in contacting ECs (PKH26 labeling of these cells evident in Fig. 2K). Heterocellular EC-MC coupling was observed regardless of which cell type was injected (75% of EC injections (n=4) and 50% of *L*MC43-tr injections (n=6) were positive for heterocellular coupling).

Cx43tr and Cx43-T154A do not support EC-induced mural cell differentiation.
To determine whether Cx43 mediated communication is both necessary and sufficient for EC-induced MC differentiation, MC43$^{-/-}$, *L*MC43-wt, *L*MC43-T154A, or *L*MC43-tr cells were co-cultured with endothelial cells, and assayed for EC-induced expression of mural cell markers (smooth muscle alpha actin (SMA) by immunocytochemistry; SMA and calponin by western blotting of total protein from whole-cell lysates). Immunocytochemistry showed that SMA was significantly increased in *L*MC43-wt cells co-cultured with ECs compared to *L*MC43-wt solo culture (Fig.3 A-C: 266$\pm$26% of control, $p<0.05$, n=6; signal normalized to number of MC nuclei). In contrast, no increase in SMA expression was observed in *L*MC43-T154A (Fig.3D-F, n=6) or *L*MC43-tr (Fig.3G-I, n=7) co-cultured with ECs compared to solo cultures. The change in SMA expression by *L*MC43-wt cells when co-cultured with ECs was accompanied by changes in cell morphology consistent with mural cell differentiation. In solo culture, *L*MC43-wt cells were devoid of obvious actin filaments (Fig. 3A) except, occasionally, at the

periphery of the cells. In contrast, *L*MC43-wt in co-culture with ECs tended to adopt an appearance with well-defined, easily detected actin filaments throughout the cell (Fig.3 B). *L*MC43-T154A and *L*MC43-tr cells in co-culture with ECs did not undergo similar morphological changes.

To corroborate the immunocytochemistry-based findings, we isolated total protein from MC solo cultures and EC-MC co-cultures and performed western blotting as described (see methods). Consistent with the immunocytochemistry data, we observed an increase in SMA as well as calponin expression in *L*MC43-wt cells when co-cultured with ECs (153±33% and 176±59% for SMA (n=7) and calponin (n=6), respectively), but not in either *L*MC43-T154A (SMA, n=4;calponin n=3) or *L*MC43-tr (SMA, n=7;calponin, n=4) cells when co-cultured with ECs (Fig. 4). Increased expression of both SMA and calponin by *L*MC43-wt cells was consistently observed in experiments comparing co-cultures to solo culture conditions (p=0.030 and p=0.023 by sign test for SMA and Calponin, respectively), although significance by one-sample t-test was not achieved (p=.077 and p=.128 for SMA and calponin, respectively).

MCs expressing Cx43-T154A or Cx43tr differentiate in response to exogenous TGF-β.

We next determined whether the inability of MCs to undergo EC-induced mural cell differentiation stemmed from inability of these cells to respond to exogenously applied TGF-β. Immunocytochemistry (Fig. 5, n=3 for $MC^{-/-}$ group and n=5 for all other groups) and western blotting experiments (Fig. 6) revealed that both *L*MC43-T154A and *L*MC43-tr cells significantly up-regulated SMA (Fig. 5,6) and calponin (Fig. 6, n=4 and n=3 for *L*MC43-T154A and *L*MC43-tr, respectively) expression in response to exogenous TGF-β treatment, suggesting that their failure to differentiate in co-culture is not likely caused by inability of these cells to activate TGF-β receptors and their downstream signaling components. However, the response of these cells to exogenous TGF-β, gauged by both immunocytochemistry and western blotting, was somewhat attenuated compared to the response of similarly treated *L*MC43wt. When compared by ANOVA these differences did not reach statistical significance (p=0.169 and 0.125 for immunofluorescence and western blotting experiments, respectively).

Discussion:

Previous studies have demonstrated that Cx45 and Cx43 are both necessary, and neither sufficient without the other, for proper development of the embryonic vasculature, but the signaling processes supported by these proteins and the mechanisms underlying their support have not been completely defined. Embryos deficient for Cx45 appear pale, anemic, and fail very early in development (E 9.5-10.5) due to inability to establish mature, functional blood vessels. Importantly, in these embryos developing blood vessels fail to recruit mesenchymal cells and induce their differentiation to form arterial vascular walls.[23] In contrast, Cx43 deficient embryos fail much later in development due to an obstruction of the right ventricular outflow tract and abnormal patterning of the

coronary vasculature caused, at least in part, by inappropriate migration, proliferation and/or differentiation of coronary vascular precursor cells.[17-19] That the aforementioned vascular defects were observed despite co-expression of Cx43 and Cx45 in adult vascular smooth muscle suggests that there are connexin-specific functions of these proteins, that their expression or regulation differs in precursor cells over the course of development, or both. Interestingly, animals expressing Cx43K258stop, which eliminates residues 258-382, develop to adulthood, suggesting that Cx43 channel function may be sufficient for vascular development provided Cx45 is also expressed.[37] A complete profile of the functional consequences of connexin expression in vascular precursor cells throughout embryonic development is currently unavailable; nonetheless, in-vitro models of cell-cell interactions important to blood vessel formation have provided a way to address some of these questions.

We previously reported use of an *in vitro* model to determine whether Cx43 (or Cx45) expression is necessary for EC-induced differentiation of MCs toward a mural cell phenotype.[3,7,26] The model system relies on use of MCs derived from Cx43 deficient mice (MC43$^{-/-}$) that differentiate to a mural cell phenotype when exogenously stimulated with TGFβ but do not so differentiate when co-cultured with ECs unless genetically modified to either re-express Cx43 (Re43 cells[7]) or to increase Cx45 expression (Re45 cells[26]). These studies demonstrated that Cx43, the predominant connexin expressed by these MCs[38], is necessary for EC-induced, TGFβ-mediated differentiation of MCs toward a mural cell phenotype, and that Cx45, when expressed at levels sufficient to support detectable EC-MC intercellular coupling, also supports differentiation. Other groups using similar approaches have also demonstrated that ECs can induce and maintain a differentiated (contractile) phenotype in a number of SMC precursors, as well as cultured SMCs, in a TGFβ- and Cx43-dependent manner.[24,39,40]

Because the most conspicuous function of connexins is to support GJIC via formation of gap junctions, it has been the prevailing view that this is the connexin-mediated function that is critical in support of EC-induced modulation of mural cell differentiation. However, in recent years it has become increasingly clear that connexins also participate in transmembrane signaling and numerous protein-protein interactions[30,41], some of which may be very relevant to EC-induced modulation of mural cell differentiation.[27,29] Studies aimed at determining whether Cx43 is critical for EC-induced mural cell differentiation utilized Cx43 knockout or knockdown strategies, which interfere with both channel dependent and independent contributions of Cx43[24]. In attempts to distinguish between channel dependent and independent mechanisms, these investigations have also made use of gap junction channel blockers such as carbenoxolone and 18α-glycyrrhetinic acid.[24,25] They found that in the presence of these compounds, EC-induced mural cell differentiation was not observed and concluded, therefore, that the critical function of connexins is, indeed, support of EC-MC intercellular communication. However, these gap junction channel blockers have non-specific effects: they are able to block all gap junction channel types irrespective of the

comprising connexin isoform and also block other types of channels. These non-specific effects make these blockers a less than ideal choice in this experimental paradigm. These limitations of previous approaches prompted us to use our previously described *in vitro* model system[7] to test mutant forms of Cx43 for their ability to support EC-induced MC differentiation to a mural cell phenotype.

Herein we tested whether channel function was necessary for EC-induced MC differentiation to a mural cell phenotype using a mutant form of Cx43, Cx43-T154A. Beahm, et al (2005) demonstrated that substitution of the threonine at position 154 (within the third transmembrane domain) with alanine results in a dominant-negative form of Cx43 that displays proper synthesis, localization, and gap junction plaque formation, but is “channel-dead” - unable to support electrical or chemical intercellular communication.[34] We observed that MCs expressing Cx43wt differentiated toward a mural cell phenotype when co-cultured with ECs, while those expressing the channel-dead Cx43-T154A did not, despite being responsive to exogenous TGF-β. These data highlight the necessity of channel function to EC-induced MC differentiation and also indicate that the presence of a normally localized carboxyl-terminal (CT) domain is not sufficient to support such differentiation.

Although the Cx43-T154A mutant results clearly demonstrated that normal localization of the Cx43 CT regulatory domain was not sufficient to support EC-induced MC differentiation, this mutant did not resolve whether this regulatory domain was necessary to the differentiation process. The permselective and gating properties of Cx43 channels are regulated, in large part, by the carboxyl terminal (CT) domain (for review see Ek Vitorin, 2013) and this region is also involved in the protein-protein interactions of this connexin.[30] To begin addressing the possible importance of this regulatory domain, we used Cx43tr257, which truncates nearly all of this domain. Our data show that this regulatory domain of Cx43 is also necessary for this protein to support EC-induced MC differentiation, and channel function per se is not sufficient to support EC-induced MC differentiation. It remains unclear whether the importance of this domain lies in regulation of channel properties, which differ between truncated and WT channel, or in supporting necessary protein-protein interactions, or both. This observation is interesting for several reasons. First, it suggests that in addition to the tubulin binding region of the CT, which is retained along with a *src* targeted tyrosine in our Cx43tr257 mutant, other parts of the Cx43 CT domain are necessary for EC-induced mural cell differentiation. Second, the fact that communication without the regulation conferred by the CT was not sufficient to support the differentiation process raises the possibility that the communicating junction may need to be regulated to a particular sub-conductive or selective state for Cx43 to support differentiation (in the absence of significant Cx45 expression). Further studies will be necessary to determine the specific sites within the CT necessary for EC-induced differentiation and to identify the signaling cascades critical to their regulation.

Finally, while both the *L*MC43-T154A and *L*MC43-tr cell types differentiated when stimulated with exogenous TGF-β, the magnitude of the response was apparently less than observed in MC43$^{-/-}$ as well as in *L*MC43-wt cells. This result suggests that TGF-β activated signaling cascades are intact and responsive in *L*MC43-T154A and *L*MC43-tr cells, but the effect of activation is regulated by Cx43, when present, in MCs. This regulation likely reflects channel-permeation independent functions of Cx43, likely through protein-protein interactions, but further studies will be necessary to resolve this issue.

In summary, our data indicate that Cx43 supports EC-induced mesenchymal cell differentiation to a mural cell phenotype in a manner requiring both heterocellular intercellular communication and the carboxyl terminal regulatory domain. Our data further suggest that intercellular communication between EC and MC is necessary for activation of TGF-β by endothelial cells whereas the CT regulatory domain of Cx43 can both enable as well as inhibit the response of MCs to TGF-β stimulation.

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Disclosures: None

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Significance:

Connexins, and the gap junction intercellular communication they support, are major components of the endothelial and smooth muscle cell signaling events critical to developmental, pathological, and regenerative processes in the vasculature. To our knowledge, the data presented herein are the first to indicate that a functional gap junction channel and the major regulatory mechanism are both necessary, but neither alone is sufficient, to support endothelial cell-induced differentiation of mesenchymal cells toward a mural cell phenotype. In light of these data, future studies aimed at exploring connexin-dependent signaling pathways in the contexts of vascular development or re-generation should consider the implications for both the gap junction channel and the regulatory domain.

Figure Legends:

Figure 1. Cx43 expression supports intercellular communication in otherwise communication deficient Cx43$^{-/-}$ MCs. Cx43$^{-/-}$ MCs (A) stained for nuclei (Hoechst dye, blue) and Cx43 (white), showed no evidence of Cx43 expression. (B-E) An MC43$^{-/-}$ cell (arrowhead) injected with a mixture of gap junction permeable NBD-m-TMA (NBD) and impermeable rhodamine dextran (Rhodex), revealed no coupling to neighboring cells (no NBD positive, Rhodex negative cells; n=10). (F) Cx43 expression in *L*MC43-wt cells was robust with numerous puncta at appositional membranes (arrows), evidence of gap junction plaque formation. (G-J) *L*MC43-wt cells were well coupled, with nearly all cells contacting the injected (arrowhead) cell positive for NBD-M-TMA but negative for Rhodex (n=15 injections, all positive for coupling). Scale bar corresponds to 100 μm.

Figure 2. Expression and function of Cx43 in *L*MC43-T154A and *L*MC43-tr cells. (A,D) Cx43-T154A (white) expression in *L*MC43-T154A cells was robust

including at appositional membranes (A, inset), indicative of gap junction formation. (B,C) *L*MC43-T154A cells were not dye coupled (n=6; arrowhead indicates injected cell), indicating Cx43-T154A gap junctions were not functional. (E,D) Cx43 expression in *L*MC43-tr could not be detected (antigenic sequence absent) by immunofluorescence or Western blot. (F-I) *L*MC43-tr cells were, however, dye coupled (n=31; arrowhead indicates injected cell). Scale bar corresponds to 100 μm. (J) ECs in co-culture with *L*MC43-tr cells form functional gap junctions with other ECs (PKH labeled; K,L) as well as MCs (L). In this example, the EC indicated in panel A was injected with NBD-M-TMA, which appeared in a neighboring MC (arrowhead in L) as well as other ECs (L). Heterocellular coupling was evident in 3 of 4 experiments where the EC was injected and 3 of 6 experiments where the MC was injected.

Figure 3. Cx43-T154A and Cx43-tr expressing MCs in co-culture with ECs do not undergo mural cell differentiation like Cx43-wt expressing MCs. (A,B) *L*MC43-wt in co-culture with ECs (B) show evidence of mural cell differentiation, forming numerous SMA fibers and overall increased SMA staining. Similar changes in expression were not observed in Cx43-T154A (D-F) or Cx43-tr (G-I) expressing MCs. The scale bar in panel A represents 100 μm and applies to all images.

Figure 4. Cx43-T154A and Cx43tr do not support EC-dependent up-regulation of mural cell markers. Total protein isolated from the indicated cell types in co-culture or not with ECs revealed upregulation of SMA and calponin in Cx43-wt expressing cells but not in Cx-deficient, nor Cx43-T154A or Cx43tr expressing MCs. Expression in co-cultures was normalized to the paired control (solo cultures of MCs only), and percent increase determined. Graphs show the average ± SEM increase across multiple cell pairs (sample sizes indicated).

Figure 5. Exogenous TGF-β stimulates upregulation of mural cell markers by MCs irrespective of connexin expression. Immunostaining of $MC43^{-/-}$ (A,B), *L*MC43-wt (C,D), *L*MC43-T154A (E,F), and *L*MC43-tr (G,H) cells, treated (B,D,F,H) or not (A,C,E,G) with TGF-β1 (1ng/ml, 48h), revealed that irrespective of connexin expression, all MCs increased SMA expression in response to exogenous TGF-β stimulation. Panel I shows the average increase in SMA; asterisks signify significance relative to paired, non-treated control. n=3 for $MC43^{-/-}$, n=5 for all other groups. Scale bar in panel A represents 100 μm and applies to all panels.

Figure 6. Increased mural cell gene expression by MCs in response to exogenous TGF-β. Total protein was isolated from $MC^{-/-}$, *L*MC43-wt, *L*MC43-T154A and *L*MC43-tr solo cultures ($6x10^5$ cells per 100 mm diameter plate) treated or not with exogenous TGF-β, and probed for SMA and calponin expression. Band intensities were determined and the percent change in treated vs. untreated determined; the mean change in expression is plotted for each cell type (sample sizes are shown in the experimental (grey) bar). Expression of both

SMA and calponin increased in response to TGF-β. Error bars represent the standard error. Asterisks signify significance relative to untreated control.

Materials and Methods:

Cells: ECs were isolated from the bovine aorta as outlined previously, grown in low glucose Dulbecco's Minimal Essential Medium (LG-DMEM, Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Gemini Bioproducts, Sacramento, CA,USA) in a humidified incubator (37°C and 5% CO_2) and studied between passages 5 and 10. Communication deficient MCs, originally isolated from day 15.5 embryonic Cx43 knockout mice[38], were transduced with lentivirus encoding Cx43wt, Cx43-T154A or Cx43tr (Cx43K258stop) and puromycin resistance; infected cells were selected and maintained in puromycin (10 μg/ml and 1μg/ml, respectively) and used between passages 3 and 10 post infection. Co-cultures of EC and MC were seeded with $3x10^5$ ECs and $3x10^5$ MCs per 100 mm plate 48 h prior to use; single cell-type cultures (EC or MC) were seeded at $6x10^5$ cells per 100 mm plate also 48h prior to use

Transfections and Transductions: The empty, replication deficient pCIG lentiviral vector and ViraPower (Life Technologies) packaging plasmids were kindly provided by Dr. Lonnie Lybarger (Bio5 Institute, University of Arizona). The pCIG vector was modified as previously described[42] to expand the multiple cloning site and provide antibiotic (puromycin) resistance for selection. The pCIG lentiviral vector was also modified by our laboratory to contain constructs encoding Cx43wt, Cx43-T154A, or Cx43tr257.

Lentiviral vectors containing the exon 2 coding sequence for Cx43 (rattus norvegicus)[43], with or without the specified mutations, were generated as follows. XbaI and XhoI endonuclease restriction sites were introduced on the 5' and 3' ends (respectively) of the Cx43 exonic sequence by PCR amplification from an existing pcDNA3 plasmid. The forward primer was GCTCTAGAATGGGTGACTGGAGTG, and the reverse (5' to 3') primer GCCGCTCGAGTTAAATCTCCAGGTC for Cx43wt and Cx43-T154A or GCCGCTCGAGTTATGATGGGCTCAG for Cx43tr. After gel purification, PCR amplicons and pCIG vector were digested with XhoI and XbaI restriction endonucleases (New England Biolabs, 3h at 37°C), and again gel purified. The lentiviral pCIG vector was treated with calf intestinal phosphatase (New England Biolabs, 1h at 37°C), gel purified again, and vector and inserts ligated using T4 ligase (Fermentas, Pittsburgh, PA, USA) for 3h at 37°C. Ligation products were introduced into DH5-α supercompetent cells (Life Technologies, Cat.# 18265-017) and selected as per the manufacturer's instructions with ampicillin. After sequencing, desired plasmids were obtained using the Qiagen Plasmid Maxi Kit (Cat. #12163; Hilden, Germany). The Cx43-T154A mutation was introduced into the Cx43 sequence using the QuickChange Site-Directed mutagenesis kit (Stratagene, San Diego, CA, USA). The primers to convert the threonine to an

alanine codon were: CGGCTTGCTGAGAGCCTACATCATCAG (forward) and CTGATGATGTAGGCTCTCAGCAAGCCG (reverse, 5' to 3').

Lentivirus was packaged by 293T cells transiently transfected (lipofectamine 2000; Life Technologies, cat.# 11668-019) with Virapower packaging plasmids and the pCIG lentiviral plasmid. Seventy-two hours after transfection, supernatants were collected and filtered through a 0.2 μm filter. MCs grown to ~50% confluence were exposed to lentiviral-containing supernatant overnight. Thereafter, the media was changed to LG-DMEM supplemented with 10% FBS and MCs were allowed 48 h before selection with puromycin (10 μg/ml overnight). After the initial selection, cells were maintained in media containing a lower dose of puromycin (1 μg/ml). After expansion over several passages, cells were assayed for Cx43 expression, localization, and dye coupling. Cells expressing Cx43 were then stored in liquid nitrogen until the time of use.

Immunocytochemistry: Cells, cultured as described above, were plated on glass cover slips at the density outlined above, scaled down to 6 well plates. After 48 h cells were rinsed with cold phosphate buffered saline (PBS), fixed with 100% Acetone at -20^{o} C, and blocked in PBS containing 3% bovine serum albumin (BSA). Cells were stained with mouse monoclonal anti-human smooth muscle α-actin antibody (Dako; (Glostrup, Denmark) diluted 1:100; 60 min at room temperature). Cells were then stained with anti-Mouse IgG antibody conjugated to a Cy3 fluorophore (Jackson Immunoresearch (West Grove, PA, USA), 1:200; 30 minutes at room temperature) and nuclei were labeled with Hoechst dye (1:5000; 10 min at room temperature). Coverslips were then mounted on glass slides using Vectashield mounting medium (Vector Laboratories, Inc. Burlingame, CA, USA), sealed, and imaged at 10, 20, or 40X on an Olympus BX51 microscope Center Valley, PA, USA) equipped with a CoolSNAP E52 camera (Photometrix, Tucson, AZ, USA) under the control of Image Pro 6.2 software (Media Cybernetics, Rockville, MD, USA). In experiments where MCs and ECs were co-cultured, ECs were pre-labeled with 5 μM SP-DiOC$_{18}$(3) (Life Technologies Cat.# D-7778) in DMSO or PKH26 (Sigma-Aldrich, St. Louis, MO, USA) as per the manufacturer's instructions. Ratios of the average fluorescence intensity value (per MC nucleus) were obtained from paired experiments containing MCs cultured alone or co-cultured with ECs. For convenience the ratios were expressed as percent of control values.

Dye coupling: *L*MC-43wt, *L*MC-43tr and *L*MC-43-T154A cells in solo culture were injected with a mixture of dyes including NBD-M-TMA[44], a small, cationic dye that is gap junction permeable, and rhodamine-dextran, a large, gap junction impermeable dye. Transfer of NBD-M-TMA, but not rhodamine-dextran, to at least one neighboring cell indicated the presence of functional gap junctions between cells and was scored as positive for coupling. Where heterocellular coupling (EC to MC) was examined, ECs were prelabeled with PKH26 (Sigma-Aldrich) to distinguish them from unlabeled MCs, and rhodamine-dextran was not included in the injected dye mixture.

Western Blot: Cells were lysed with Laemlli buffer and total protein collected. Protein concentration was determined using the BCA assay. Thereafter, a 12% PAGE gel was loaded at 10 µg/lane for MCs and 20 µg/lane for 1:1 EC:MC co-cultures to allow direct comparison with MC-only data. (As previously published (Hirschi, 2004), we operated under the assumption that half of the protein from co-culture samples was contributed by MCs so quantitative analysis of 2X protein allows direct comparison with MC-only data). Proteins were separated in an electric field, transferred to a nitrocellulose membrane and stained for SM specific α-actin (Dako, 1:2500) and calponin (1:2000) (Millipore, Billerica, MA, USA). Chemiluminescent techniques were employed to quantify protein expression (BioRad Chemidoc XRS, Hercules, CA, USA)).

Cells, plated at densities outlined above, were cultured alone (with or without TGF-β1(1ng/ml)-Calibiochem) or co-cultured with ECs for 48h in LG-DMEM containing 2% FBS. After this 48 h treatment period, total cell protein was isolated for determination of SM α-actin and calponin expression. All experiments were performed at least three times.

Statistical Analysis: Single sample t-scores were employed to determine whether SM α-actin expression was increased by addressing whether the ratio of treated to control was different from 1. Single sample z scores were used when the sum test was employed. When multiple ratios were simultaneously compared one-way analysis of variance (ANOVA) tests were performed. In all cases significance was set at the $p \leq 0.05$.

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Fig. 1

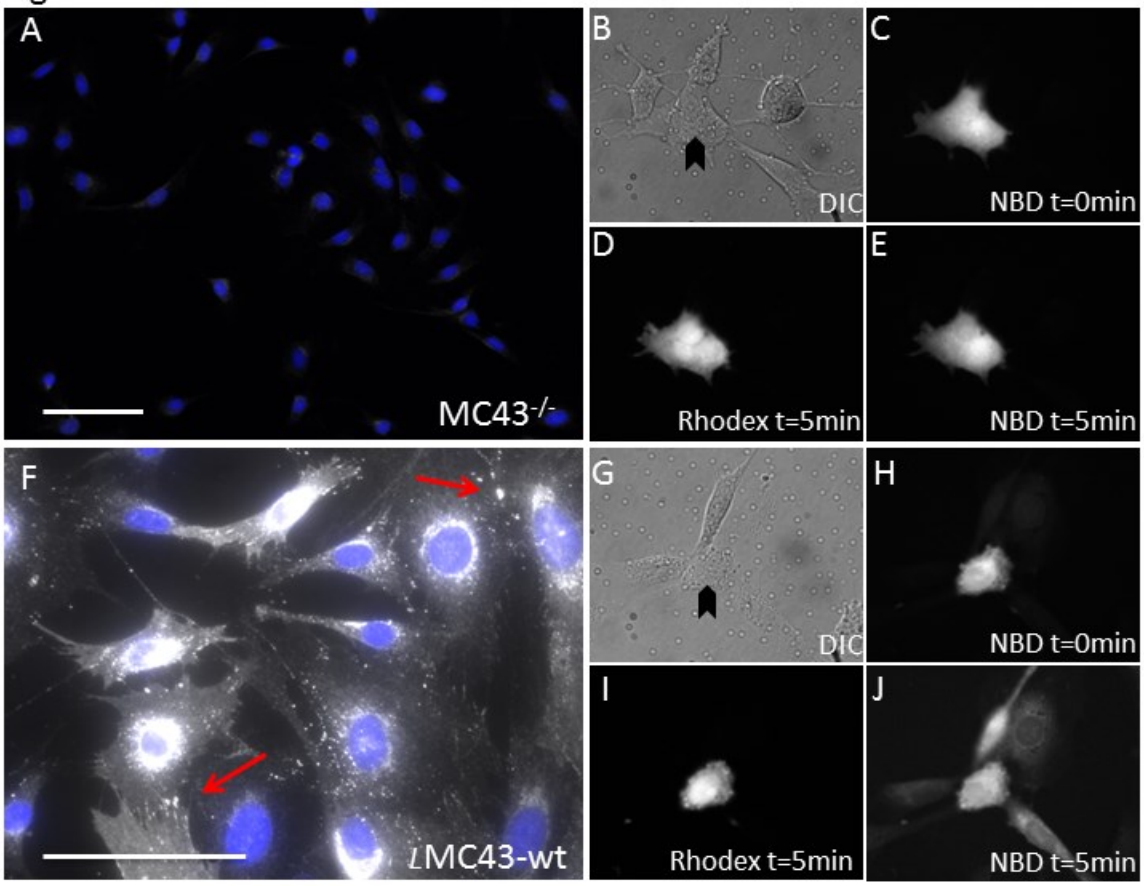
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Fig. 2

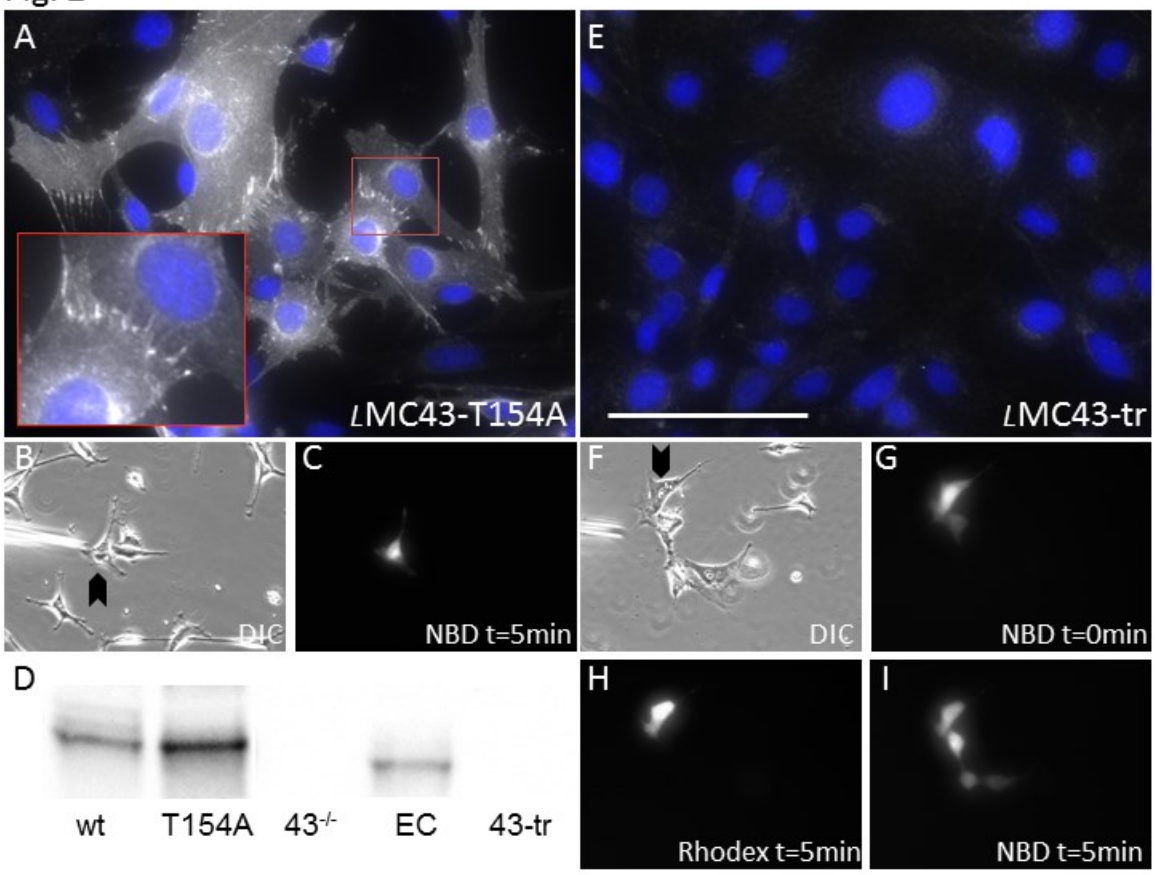
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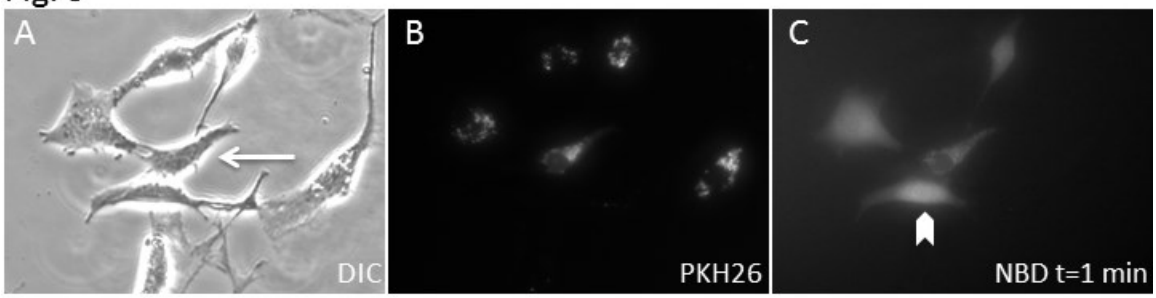
Fig. 3



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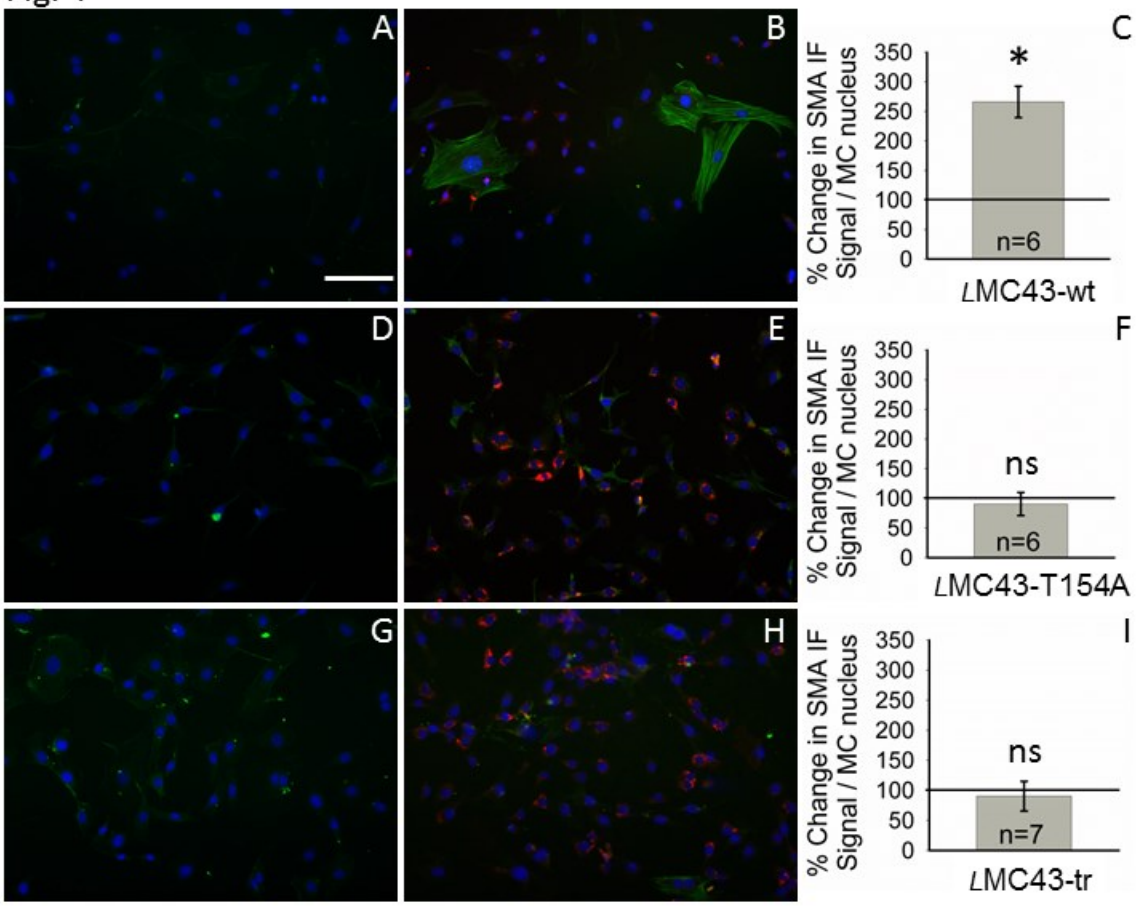
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Fig. 5

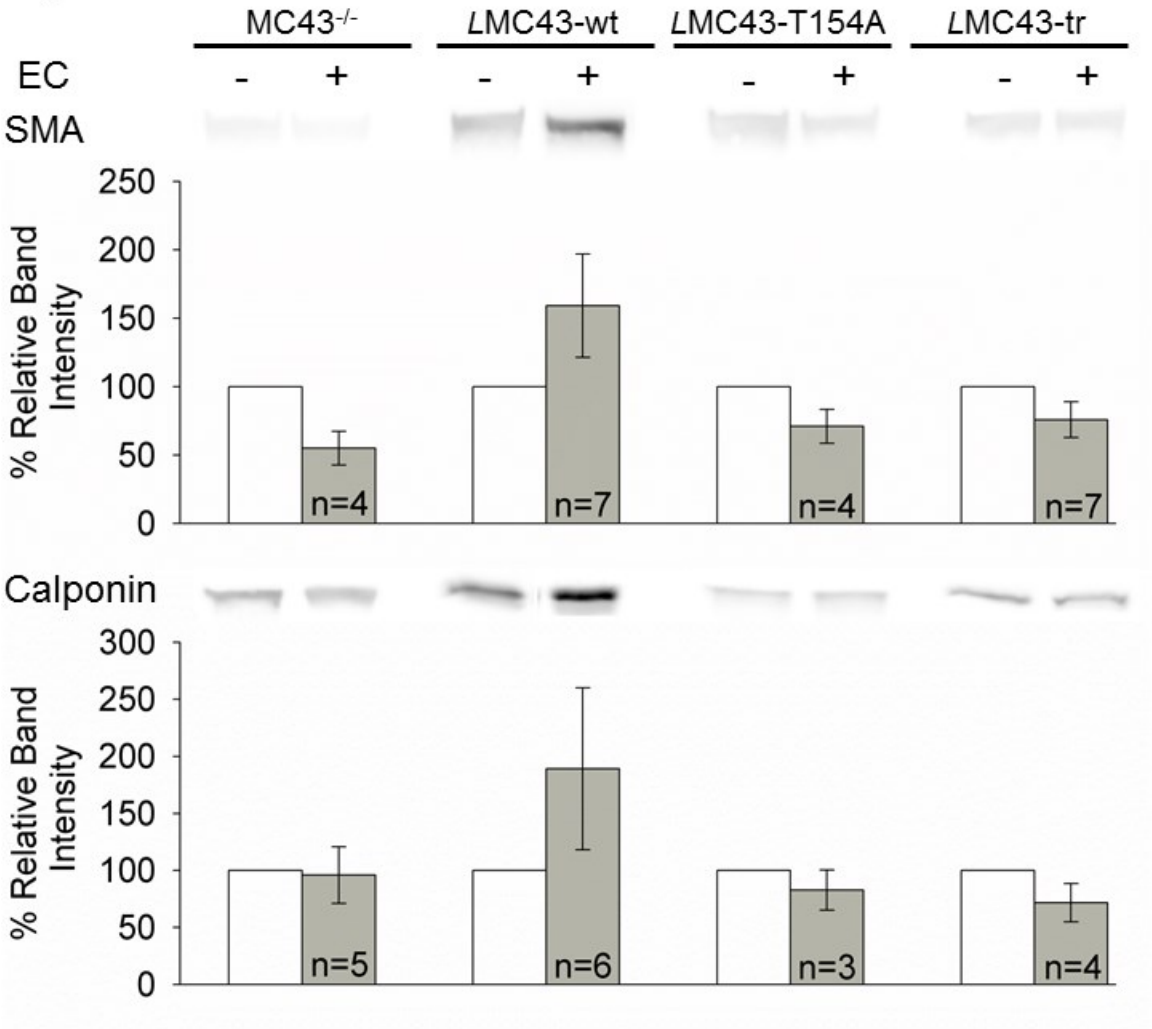
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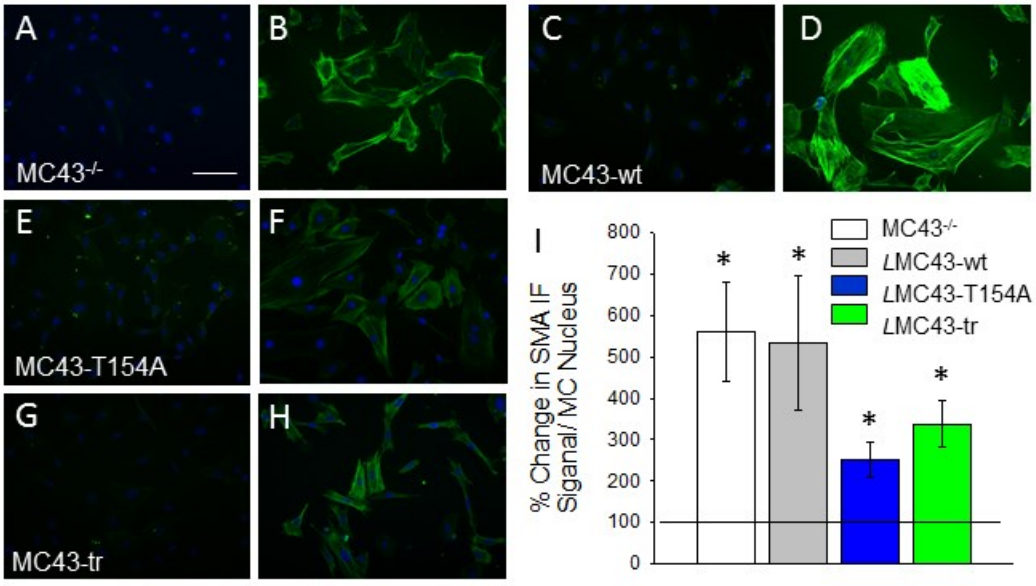
Fig. 6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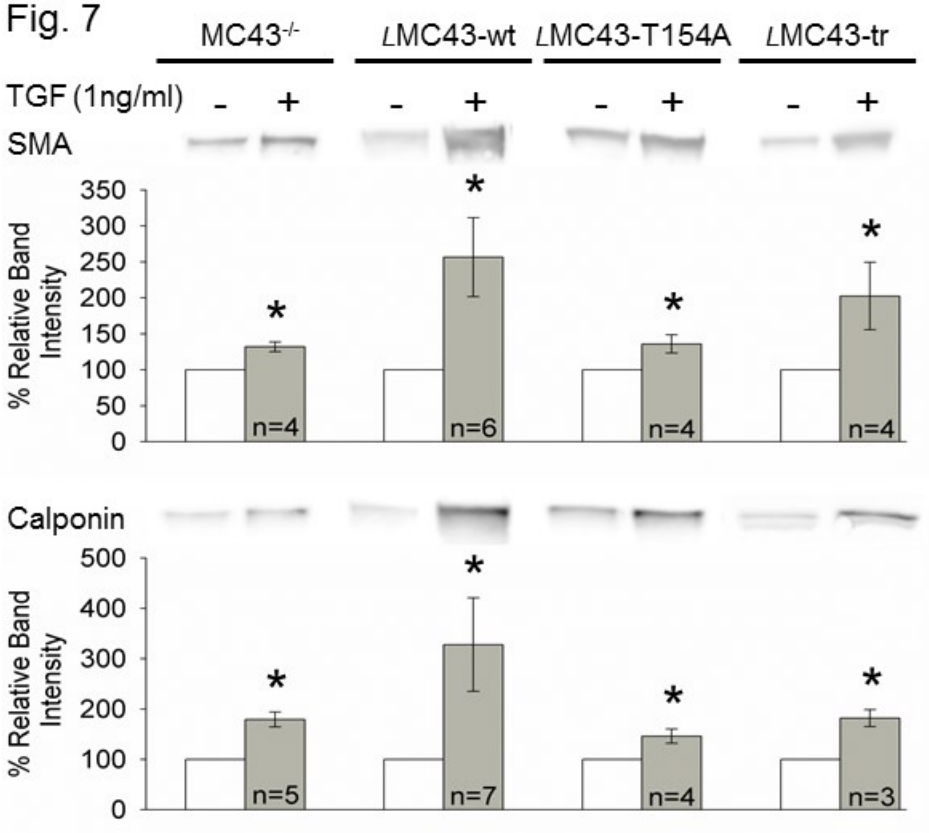
LMC43-tr

Fig. 7


MC43-/-
LMC43-wt
LMC43-T154A
LMC43-tr
TGF (1ng/ml) - + - + - + - +
SMA
% Relative Band Intensity
350
300
250
200
150
100
50
0
n=4
n=6
n=4
n=4
Calponin
% Relative Band Intensity
500
400
300
200
100
0
n=5
n=7
n=4
n=3

APPENDIX B
(Submitted for Publication at *Development*)

MAPK TARGET SITES IN CX43 ARE NECESSARY FOR ENDOTHELIAL CELL-INDUCED MESENCHYMAL CELL DIFFERENTIATION TOWARD A MURAL CELL PHENOTYPE

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Abstract:

Objective- Endothelial cell (EC)- induced mesenchymal cell differentiation toward a mural cell phenotype is TGF-β mediated, requires that MCs express connexin 43 (Cx43) or Cx45 at levels sufficient to support gap junction-mediated intercellular communication, and for Cx43 requires the carboxyl terminal regulatory domain. Which regulatory sites in the CT are necessary and whether other vascular connexins lacking similar regulation can support mural cell differentiation are investigated here.

Approach- A Cx43-deficient mesenchymal cell line, previously demonstrated to be incapable of undergoing EC-induced mural cell differentiation without re-expression of Cx43 or increased Cx45 expression, was used to evaluate MAPK or PKC site mutants of Cx43 and Cx40 for their ability to support EC-induced mural cell differentiation. Increased expression of mural cell marker proteins, evaluated using immunocytochemistry and western blotting techniques, indicated differentiation. Cx43 MAPK mutants, with serine residues 255, 279 and 282 mutated to alanine, did not support EC-induced upregulation of mural cell genes despite supporting intercellular communication and response to exogenous TGF-β. The PKC mutant of Cx43 with serine 368 mutated to alanine supported mural cell differentiation. Cx40, whose channel function is not regulated by MAPK or PKC, also failed to support EC-induced mural cell differentiation.

Conclusions-These data demonstrate that MAPK- but not PKC- dependent regulation of Cx43 is necessary for Cx43 to support EC-induced mural cell differentiation. Cx40 did not support this process despite supporting gap junction-mediated intercellular communication.

Key Words: mural cell differentiation, Connexin, MAPK

Introduction:

During angiogenesis endothelial cells (ECs) secrete PDGF-B, which acts as a chemoattractant and mitogen that induces the targeted migration of mesenchymal cells (MCs) toward endothelial cell tubes[1-3]. Upon contact and gap junction formation (Cx45 and/or Cx43), the MCs differentiate toward a mural cell phenotype, the hallmark features of which includes (among other things) upregulation of contractile genes, such as smooth muscle alpha actin (SMA), transgelin (SM22 alpha), calponin, myosin heavy chain (MHC), and others[4]. The differentiation of MCs toward a mural cell phenotype is complex, involving multiple factors, but is critically dependent upon activation of transforming growth factor beta (TGF-β). The effects of TGF-β in this setting are thought to be mediated via type I activin-like kinase (ALK) receptors, TβRII receptors and canonical pSmad 2/3 signaling pathways[5], although evidence for the involvement of non-canonical pathways (such as Rho, Rac, and MAPK) is also emerging.[6,7]

Connexin expression is necessary for normal vascular development and homeostasis. Of the more than twenty connexin isoforms identified to date, only four (Cx37, Cx40, Cx43, and Cx45) are expressed in the mature arterial vasculature where their pattern of expression varies depending on the vascular bed, tissue/ cell type, and proliferative (or quiescent) state.[8-12] Quiescent endothelial cells in mature arteries express mainly Cx40 and Cx37, whereas proliferating and microvascular endothelial cells also express Cx43. MCs and smooth muscle cells (SMCs) predominantly express Cx43 and low levels of Cx45[11,12,13]; however, in some vessel beds these cells can also express Cx37.[14,15] Loss of either Cx40 or Cx37 alone does not lead to embryonic lethality or failure of vascular development [16,17], but genomic deletion of either Cx45 or Cx43 results in lethality due largely to defects in vascular development. Cx45 deficient animals die in the early stages of embryonic development due to inability to form mature blood vessels. Importantly, in these animals vasculogenesis appears to occur normally, but endothelial cell tubes do not acquire a vessel wall.[18] Interestingly, deletion of Cx45 specifically in differentiated smooth muscle cells produces no obvious vascular defects[13]. Cx43 deficient animals exhibit abnormal patterning of the coronary vasculature and die at birth due to obstruction of the right ventricular outflow tract. The cardiovascular phenotypes found in Cx43 deficient animals have been linked to the abnormal migration and/or differentiation of proepicardial and neural crest cells, the two cell populations involved in formation of the coronary vasculature.[19-21] Taken together, these data suggest that connexins 43 and 45 are essential to the angiogenic and remodeling phases of vascular development and their contributions are only partially overlapping, with neither connexin able to fully compensate for loss of the other.

Connexins contribute to cell functions such as coordinated contraction and secretion, and cell migration, proliferation and differentiation in a connexin-, cell- and tissue-specific fashion via one, or a combination, of three basic signaling mechanisms: gap junction mediated intercellular signaling, gap junction

hemichannel mediated transmembrane signaling, and intracellular signaling through interaction with scaffolding and signaling cascade components. Using an *in vitro* model of EC-induced mural cell differentiation that recapitulates the *in vivo* process, we showed previously that without Cx43 or levels of Cx45 expression sufficient to support detectable gap junction-mediated intercellular communication with ECs, MCs fail to acquire a mural cell phenotype when co-cultured with ECs, although these connexin "deficient" MCs do differentiate when directly stimulated with TGF-β.[4,22,23] We have subsequently begun to examine the mechanistic basis for Cx43-supported, EC- and TGF-β -dependent MC differentiation. Data from our laboratory (Angelov, unpublished results) indicate that the expressed Cx43 must be able to form functional channels, although it is unclear whether intercellular signaling is actually required or if channel functionality is indicative of a specific connexin conformation able to support intracellular signaling through protein-protein interactions. We also found (Angelov, unpublished results) that the carboxyl terminal (CT) domain must be present for Cx43 to support EC-dependent MC differentiation, channel function without this regulatory domain is not sufficient. These results prompted us to hypothesize that regulatory sites within the CT are necessary for Cx43 to support EC-induced mural cell differentiation. Previous studies have implicated Mitogen Activated Protein Kinase (MAPK) and Protein Kinase C (PKC) in regulation of Cx43 channel function and cell response to growth factors such as PDGF and TGF-β.[24-29] Thus, we test here whether MAPK and PKC sites in the Cx43 CT, known to regulate Cx43 channel function, are necessary for Cx43 to support EC-induced MC differentiation, and whether a connexin whose channel function is not regulated by these signaling cascades could substitute for Cx43 in supporting mural cell differentiation. We show that MAPK target sites (S255, 279, and 282) are necessary, while PKC-dependent regulation at S368 is not necessary, for Cx43 to support EC-induced MC differentiation. Further we show that Cx40 does not support MC differentiation.

Results:

In recent studies (Angelov, unpublished results), we showed that for Cx43 to support EC-induced MC differentiation towards a mural cell phenotype, the Cx43 must be able to form functional channels and its CT domain had to be present. The necessity for the CT could reflect its interactions with the pore-forming domains of Cx43 (specifically the cytoplasmic loop and or amino terminus) to regulate channel function, and/or its interactions with other proteins in the cytoplasm to modulate intracellular signaling cascades and response to TGF-β. Both types of interaction are modulated by phosphorylation-dependent processes. Here we test the hypothesis that specific MAPK and PKC regulatory sites in the CT of Cx43 are necessary for this connexin to support EC-induced mural cell differentiation and determine whether a connexin whose channels are not regulated by these kinases, Cx40, can substitute for Cx43 in supporting EC-induced differentiation.

MAPK-targeted serines 255, 279 and 282 are necessary for Cx43 to support EC-induced mural cell differentiation.

MC43$^{-/-}$ were infected (lentivirus: *L*) to express Cx43 with alanine substitutions at serines 255, 279, and 282 (*L*MC43-S3A). These cells were co-cultured with ECs and evaluated for EC-induced mural cell differentiation. As reported previously[4], Cx43 is not detected in MC43$^{-/-}$ and these cells are not detectably dye coupled (**Fig. 1 supplement)**; in contrast, Cx43 was readily detected in MC43$^{-/-}$ cells infected to express wild-type Cx43 (*L*MC43) and these cells were dye coupled (**Fig. 1 supplement**), as expected from previous studies where Cx43 was introduced by different methods[4,30]. Cx43-S3A expressed well in *L*MC43-S3A cells, forming readily detectable gap junction plaques that support dye-coupling (**Fig. 1**). Immunocytochemistry for SMA expression in solo *L*MC43 and EC-*L*MC43 co-cultures showed, as expected[4], significant up-regulation of SMA (289±32% increase in fluorescence intensity, n=7; **Fig. 2A-C**) in the co-culture setting. In contrast, Cx43-S3A expressing cells were not comparably induced by ECs to upregulate SMA (**Fig. 2D-F**; fluorescence intensity 89±28% of solo culture value, n=3). Nevertheless, the *L*MC43-S3A up-regulated SMA in response to exogenous TGFβ stimulation (591±149% increase above solo culture, n=3; **Fig. 2G-I**). Additionally, whereas *L*MC43 in co-culture with ECs tended to develop well defined and oriented SMA fibers, previously reported as a feature of mural cell differentiation[31], the *L*MC43-S3A cells failed to develop such fibers (**Fig.2E**, n=3) unless exogenously stimulated (1 ng/ml, 48 hours) with TGFβ (**Fig. 2H and supplemental Fig.7**). A similar overall outcome was observed when calponin was used as a marker of mural cell differentiation (data not shown).

To corroborate the findings obtained with immunocytochemistry approaches, we used western blotting techniques to assess the expression of SMA and calponin in EC- *L*MC43-S3A co-culture vs. *L*MC43-S3A solo cultures. Although expression of both these mural cell markers in EC-*L*MC43 co-cultures is consistently elevated relative to solo cultures (153±32% for SMA (n=7) and 176±59% for calponin (n=6), significant by sign test, p=0.029 and 0.023, respectively), neither protein was upregulated in EC-*L*MC43-S3A co-cultures (**Fig. 3A;** 83±24% (n=4) and 70±20% (n=4) of solo culture control for SMA and calponin, respectively). A trend towards increased expression of both proteins was observed in *L*MC43-S3A cells exogenously stimulated with TGF-b (**Fig. 3B;** compared to untreated controls 3 of 4 experiments showed increased SMA expression: 193±48%, p=0.075; and 4 of 4 experiments showed increase calponin expression: 149±25%, p=0.07). We further examined which of these MAPK-targeted serines is required for Cx43 to support EC-induced MC differentiation, serine 255, targeted by BMK1[32] or serines 279 and 282, targeted by ERK1/2 [33]. These experiments suggest that both the BMK1- and ERK1/2-targeted sites are required (see supplemental data **Figures** 2-4).

Together, the immunocytochemistry, cell morphology and western blot data strongly suggest that MAPK targeted serines in the CT of Cx43 are required for Cx43 to support EC-induced MC differentiation, despite the ability of this mutant to form functional gap junctions.

Serine 368, a PKC target, is not necessary for Cx43 to support EC-induced mural cell differentiation.

PKC is activated in EC-induced MC-differentiation during MC recruitment (via PDGF) and gap junction formation between the two cell types. PDGF and PKC are known modulators of Cx43 channel function, but whether a primary target of PKC in Cx43, S368, is critical to the EC-induced MC-differentiation process has not been determined. To test this possibility, we determined whether MC43-S368A cells, which form functional gap junctions with each other[34] and with ECs (supplemental **Fig.** 5), up-regulated mural cell markers when co-cultured with ECs. **Fig. 4** shows that, indeed, EC-induced mural cell differentiation is supported by Cx43-S368A, suggesting that phosphorylation at this site by PKC is not necessary for this process.

Cx40 does not support EC-induced mural cell differentiation.

Cx40, which is commonly expressed in arterial ECs, forms channels not regulated by PDGF-activated signaling cascades or PKC. In silico examination of the Cx40 sequence reveals an absence of any canonical MAPK consensus sites (PXS/TP). $Cx43^{-/-}$ MCs transfected with mCx40 express the mRNA and protein well, target the protein to appositional membranes, and form functional gap junctions with BAECs (Supplement **Figure 6D**). Nonetheless, Cx40 did not support EC-induced mural cell differentiation. **Figure 5** shows by Western blotting and immunocytochemistry that MC40 cells do not upregulate mural cell markers when co-cultured with ECs.

Discussion:

We previously reported that cell contact and gap junction formation by Cx43 or Cx45 are necessary and sufficient for EC-induced MC differentiation toward a mural cell phenotype[4,22]. The potential mechanisms whereby connexin expression supports mural cell differentiation remain largely unexplored, but include, in addition to intercellular signaling via gap junctions, transmembrane signaling through connexons, and intracellular signaling through protein-protein interactions, and combinations of these mechanisms. Recent studies suggested that support of EC-induced mural cell differentiation by Cx43 required the Cx43 be able to form a functional gap junction channel and include the CT domain (Angelov, unpublished observations). The CT domain confers many connexin-specific regulatory properties as well as unique protein binding interactions, reflecting the low sequence homology between connexins in CT domain. To elucidate some of the signaling cascades involved in Cx43-supported EC-induced differentiation, we investigated the necessity for MAPK and PKC target sites. Our data indicate that while serine 368, targeted by PKC, is dispensible for EC-induced differentiation, MAPK targeted serines 255, 279, and 282 are necessary for this process.

The CT domain of Cx43 (rattus norvegicus and mus musculus) contains three canonical (PxS/TP) MAPK consensus sites (around serines 255, 279, and 282),

two additional serines followed by prolines (residues 262 and 273) that may be targeted by members of the MAPK family, and multiple additional serines/threonines predicted as potential targets for phosphorylation, including by PKC at S368. Phosphorylation at the canonical MAPK sites, at S279 and S282 by Erk1/2 and at S255 by MAPK7, decreases gap junction-mediated intercellular communication (GJIC) and mutation of these sites to alanine eliminates EGF modulation of GJIC.[24,35,36] Phosphorylation at one or more of the MAPK sites, regulates smooth muscle cell phenotype and cell migratory behavior in response to chemical agents and growth factors.[6,25-27] The role of the canonical MAPK sites in Cx43 in MC behavior and ultimately SMC determination has not been examined. Thus, we determined whether alanine substitution for serine at positions 255, 279 and 282 altered the ability of Cx43 to support EC-induced mural cell differentiation. We found that MCs expressing Cx43-S3A (and likely Cx43-S255A or Cx43-S279,282A) were not induced to a mural cell phenotype by co-culture with ECs, suggesting these serines are necessary for Cx43 to support this process. It should be noted, however, we cannot exclude the possibility that simply mutating these serine residues to alanine, rather than preventing their phosphorylation, caused a conformational change in the Cx43CT that interfered with EC-induced (but not TGF-β induced) differentiation. Additional studies will be necessary to determine whether the Cx43-S3A protein fails to support differentiation due to inability of the gap junction channels to be closed by MAPK-dependent phosphorylation, altered interaction of Cx43 with other proteins involved in mural cell differentiation, or even altered Cx43-mediated transmembrane signaling.

Our data do not provide insight on which kinases target these residues during EC-induced mural cell differentiation. ERK1/2 has been shown to target the S279,282 residues[33], MAPK5 targets S255[32], but other kinases may phosphorylate Cx43 at these and the other possible MAPK sites. Phosphorylation site prediction programs (e.g.: http://gps.biocuckoo.org/online.php) indicate a high probability of phosphorylation at the five target serines by MAPK isoforms 1, 3, 7, 8, 10, 11, 12, 13 and 14 as well as CDKs 5 and 4, with MAPK8 (a JNK) having the highest probability at all sites. Serines 262 and 273 have the most restricted profile of targeting kinases, with very low probability of phosphorylation by MAPK 1, 3 or 7, or CDKs). MAPK isoforms tend to be activated by different stimuli and can be involved in modulating alternate cellular processes.[37] Members of the ERK group of MAPKs (1, 3 and 7) tend to be activated by growth factors, phorbol esters, G protein coupled receptors and microtubule disorganization.[38] Members of the p38 and JNK groups of MAPKs tend to be activated by cytokines and environmental stress and are often important in regulating cytokine expression and immune function.[39]

In the co-culture model used herein the profile of activated MAPKs over the 48 h induction period is unknown. However, both PDGF and TGF-β (the factors involved in recruitment and differentiation of mesenchymal cells, respectively) are involved in Cx43-supported, EC-induced mural cell differentiation and both signaling molecules activate members of the MAPK family. In recent years a

number of studies have reported that MAPK signaling pathways are able to modulate TGF-β signaling in both a Smad-dependent and Smad-independent manner.[7] Thus, a potential role for Cx43 and MAPK signaling pathway crosstalk in the context of TGF-β dependent differentiation should be explored further. It is also unclear whether the apparent necessity for MAPK phosphorylation of Cx43 during mural cell differentiation is unique to certain types of EC-MC interaction or whether it is a more broadly occurring phenomenon. Goerke et al[40] reported that human mesenchymal stem cells differentiate toward a smooth muscle cell phenotype when co-cultured with human endothelial progenitor cells in a cell contact and ERK-dependent manner, but independent of GJIC (as detected by Lucifer yellow scrape loading techniques). Their conclusion that differentiation does not require GJIC suffers from insensitivity of their technical approach. Lucifer Yellow does not permeate Cx45 gap junction channels well, and low levels of coupling by Cx45 or Cx43 may not be detected by this technique. In addition, they did not determine whether Cx45 was expressed in their MC population and did not demonstrate absence of gap junction plaques. Thus, it remains uncertain from the Goerke study whether gap junctions were present, although levels of dye coupling were certainly reduced by their treatments. Nonetheless, the Goerke study result is provocative because it may indicate that differentiation mechanisms differ depending on the source of (precursor) endothelial and mesenchymal cell populations. Clearly, additional studies will be necessary to address these issues and to determine the contexts in which various differentiation mechanisms are relevant.

In addition to activating MAPK-signaling, many growth factors, including PDGF, activate PKC-dependent signaling. This kinase similarly regulates Cx43 channel function[41], phosphorylating Cx43 on S368 (and possibly other serines) to reduce GJIC.[42] We show here that mutation of this serine to alanine had no obvious impact on Cx43's ability to support EC-induced MC differentiation. We also determined whether Cx40 could support EC-induced MC differentiation. Cx40 is commonly expressed in arterial endothelium, but this channel's function is not obviously regulated by either PKC[47] or MAPKs (absence of appropriate consensus sequences). Thus, if intercellular communication were all that was required, this connexin would be expected to support EC-induced MC differentiation. Our results indicate that despite supporting gap junction formation and GJIC between neighboring ECs and MCs, this connexin did not support EC-induced mural cell differentiation. This observation is interesting because it re-affirms the notion that vascular connexins serve unique as well as overlapping functions with other connexins. Further, this result suggests that GJIC between ECs and MCs (at least as indicated by intercellular transfer of Lucifer yellow dye or NBD-M-TMA), in the absence of other aspects of connexin-specific function, is not sufficient to support EC-induced mural cell differentiation.

The structural and functional aspects of Cx-specificity that enable EC-induced mural cell differentiation are not necessarily obvious, or even identical, as suggested by the different points in development for lethality of Cx45 vs. Cx43 deficiency. For example, the MAPK targeted serines that we determined critical

for Cx43-dependent, EC-induced, differentiation are not present in the CT of Cx45, yet this connexin also supports EC-induced differentiation. All the connexins tested in the current study support GJIC between EC and MC, even the mutants, yet only Cx45 and Cx43 with or without serine 368 supported EC-induced mural cell differentiation. Together, the data suggest that connexins enable EC-induced mural cell differentiation in a manner that involves at least two of the possible mechanisms these proteins use to mediate signaling – intercellular signaling through gap junction channels and intracellular signaling through protein-protein interactions. Interestingly, transient interruption of one or both of these contribution strategies may be key to EC-induced MC differentiation. Previous studies showed that the proliferative response of Cx43 expressing MCs to EGF involves MAPK activation, phosphorylation at serines 255, 279 and 282, and decreased GJIC. Mutation of the MAPK targeted serines to alanines interfered significantly with these responses to EGF[35,36]. Mutation of these same sites in the current study associated with inability of Cx43 to support EC-induced differentiation, raising the intriguing possibility that perhaps temporary reduction (since communication competent Cx43 is required; Angelov, unpublished results) in GJIC may be a necessary step in this process. Clearly, blood vessel formation is complex and relies on a number of signaling events that exhibit a high degree of spatiotemporal specificity. The role of connexins in this process is therefore likely to be complex and multifaceted as well. It is likely that both regulated GJIC and protein-protein interactions are involved in EC-induced mural cell differentiation and having only one aspect of Cx function is not sufficient to support the process.

In summary, the data presented herein demonstrate that MAPK targeted residues (S255,279, and 282) in the CT domain of Cx43 are necessary and the PKC targeted serine residue at position 368 dispensable for Cx43 to support EC-induced differentiation of mesenchymal cells toward a mural cell phenotype. Additionally, our data demonstrate that unlike Cx43 and Cx45, Cx40 cannot support EC-induced mural cell differentiation.

Figure Legends:

Figure 1: Expression and function of Cx43-S255A, S279A, S282A (Cx43-S3A).

(A) Expression of Cx43-S3A is readily detected in *L*MC43-S3A cells, with gap junction plaques (inset) evident at appositional membrane. Cx43-S3A gap junctions consistently supported dye coupling (4 of 4 injected cells showed coupling to multiple neighbors). In this example (B-E), a mixture of NBD-M-TMA and rhodamine-dextran was injected into the indicated cell (arrow in B). NBD-M-TMA diffused from that cell (C) to neighboring cells (arrowheads) over time (E), while the gap junction impermeable rhodamine-dextran remained in the injected cell (D). The scale bar in panel A represents 100 μm; panels B-E were imaged as ½ this magnification.

Figure 2: Cx43-S3A does not support EC-induced mural cell differentiation.

(A-C) *L*MC43 cells in solo culture (A) show little evidence of SMA (green) expression, but in co-culture with ECs (DiOC-red) show obvious SMA filaments (B). Across multiple experiments, SMA expression is increased in the co-culture setting (C). Similar changes in SMA expression and cell morphology were not observed in *L*MC43-S3A cultures (D-F). However, following exposure to exogenous TGF-β, SMA expression was observed in *L*MC43-S3A cells (G-I). The scale bar in panel A represents 100 μm and applies to all images.

Figure 3: Expression of mural cell markers is by *L*MC43-S3A is unaffected by co-culture with ECs but up-regulated by TGF-β.

(A) SMA and calponin expression levels were not detectably altered in total protein isolated from *L*MC43-S3A in co-culture with ECs vs. solo culture. In contrast, expression of these mural cell markers was modestly increased in *L*MC43-S3A cultures exposed to exogenous TGF-β (B). Sample sizes are shown below the bars. Expression of both SMA and calponin increased in response to TGF-β, though the effect was smaller in *L*MC43-S3A than previously reported for *L*MC43 cultures. Error bars represent the standard error.

Figure 4: Cx43-S368A supports EC-induced MC differentiation.

(A)Total protein isolated from MC43-S368A in co-culture with ECs (EC+S368A lane) vs. solo culture (S368A lane) revealed an EC-induced increase in expression of mural cell markers in the co-culture setting. Total protein isolated from smooth muscle cells served as a positive control (SMC lane). (B and C) Immunocytochemistry experiments (n>3) confirmed that expression of smooth muscle alpha actin increases in MC43-S368A upon co-culture with endothelial cells. Cells were stained with Hoescht dye to reveal nuclei as well as SMA, SMA filaments are obvious only in panel C. The scale bars in panels B and C represent 25 μm.

Figure 5: Cx40 does not support EC-induced mural cell differentiation.

EC-induced upregulation of mural cell markers in MC40 cells was not observed by western blotting (A) or immunocytochemistry approaches (B). Data representative of >3 western blot experiments and 2 immunocytochemistry experiments. In B, PKH26 panels show that ECs are present in these co-cultures, but mural cell marker panels show no evidence of EC-induced protein expression. Scale bar in B applies to all panels and represents 25 μm.

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Materials and Methods:

Cells: ECs were isolated from bovine aorta as outlined previously[43], grown in low glucose Dulbecco's Minimal Essential Medium (LG-DMEM, Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Gemini Bioproducts, Sacramento, CA,USA) in a humidified incubator (37°C and 5% CO_2) and studied between passages 5 and 10. Communication deficient MCs ($MC43^{-/-}$), originally isolated from day 15.5 embryonic Cx43 knockout mice[30], were transduced with lentivirus encoding wild-type Cx43 (*L*MC43 cells) or Cx43-S255A,S279A,S282A (*L*MC43-S3A) and a puromycin resistance gene. Infected cells were selected and maintained in puromycin (10 μg/ml and 1μg/ml, respectively) and used between passages 3 and 10 post infection.

MCs expressing Cx43-S255A (MC43-S255A) or Cx43-S279,282A (MC43-S2A) or Cx43-S368A (MC43-S368A) were made as previously described[34-36] and generously provided by Dr. Alan F. Lau. MCs expressing mCx40 were made by transfecting (using lipofectamine) $MC43^{-/-}$ cells with the pCI mammalian expression vector encoding mCx40 (MC40) and the puromycin resistance gene. MC43-S255A, MC43-S2A, MC43-S368A and MC40 cell lines were maintained under antibiotic pressure and used at passage numbers less than 20.

Co-cultures of EC and MC were seeded with $3x10^5$ ECs and $3x10^5$ MCs per 100 mm plate 48 h prior to use; cultures containing only a specific MC cell-type (solo cultures) were seeded at $6x10^5$ cells per 100 mm plate also 48h prior to use.

Lentiviral vector, Transduction and Transfection:

The empty, replication deficient pCIG lentiviral vector, modified as previously described[44] to expand the multiple cloning site and provide antibiotic (puromycin) resistance for selection, and ViraPower (Life Technologies) packaging plasmids were kindly provided by Dr. Lonnie Lybarger (Bio5 Institute, University of Arizona). The pCIG lentiviral vector was further modified to contain constructs encoding wild-type Cx43 or Cx43 with serine to alanine substitions at positions 255, 279, 282 (Cx43-S3A).

Lentiviral vectors containing the exon 2 coding sequence for Cx43 (rattus norvegicus)[44] with or without the specified mutations (255(AGC to GCT), 279(TCG to GCT) and 282(TCT to GCT)-introduced with site directed mutagenesis kit, Invitrogen), were generated as follows. XbaI and XhoI endonuclease restriction sites (sites underlined in following primer sequences) were introduced on the 5' and 3' ends (respectively) of the Cx43 exonic sequence by PCR amplification from existing pcDNA3 plasmid with or without the specified mutations. The forward primer was GCTCTAGAATGGGTGACTGGAGTG, and the reverse (5' to 3') primer GCCGCTCGAGTTAAATCTCCAGGTC. After gel purification, PCR amplicons and pCIG vector were digested with XhoI and XbaI restriction endonucleases (New England Biolabs, 3h at 37°C), and again gel purified. The lentiviral pCIG vector was treated with calf intestinal phosphatase (New England Biolabs, 1h at 37°C), gel purified again, and vector and inserts ligated using T4 ligase

(Fermentas, Pittsburgh, PA, USA) for 3h at 37°C. Ligation products were introduced into DH5-α supercompetent cells (Life Technologies, Cat.# 18265-017) and selected as per the manufacturer's instructions with ampicillin. After sequencing, desired plasmids were obtained using the Qiagen Plasmid Maxi Kit (Cat. #12163; Hilden, Germany).

Lentivirus was packaged by transient transfection (lipofectamine 2000 Life Technologies, cat.# 11668-019) of 293T cells with Virapower packaging plasmids and the pCIG lentiviral plasmid. Seventy-two hours after transfection, supernatants were collected and filtered through a 0.2 μm filter. MCs grown to ~50% confluence were exposed to lentiviral-containing supernatant overnight. Thereafter, the media was changed to LG-DMEM supplemented with 10% FBS and MCs were allowed 48 h before selection with puromycin (10 μg/ml overnight). After the initial selection, cells were maintained in media containing a lower dose of puromycin (1 μg/ml). After expansion over several passages, cells were assayed for Cx43 expression, localization, and dye coupling. Cells expressing Cx43 were then stored in liquid nitrogen until the time of use.

Immunocytochemistry: Cells, cultured as described above, were plated on glass cover slips at the density outlined above, scaled down to 6 well plates. After 48 h cells were rinsed with cold phosphate buffered saline (PBS), fixed with 100% Acetone at -20^{o} C, and blocked in PBS containing 3% bovine serum albumin (BSA). Cells were stained with mouse monoclonal anti-human smooth muscle α-actin antibody (Dako,#M0851 (Glostrup, Denmark) diluted 1:100-1:300; 60 min at room temperature). Cells were then stained with anti-Mouse IgG antibody conjugated to a Cy3 fluorophore (Jackson Immunoresearch (West Grove, PA, USA), 1:200 to 1:500; 30 minutes at room temperature) and nuclei were labeled with Hoechst dye (1:5000; 10 min at room temperature). Coverslips were then mounted on glass slides using Vectashield (Vector Laboratories, Inc. Burlingame, CA, USA) or MOWIOL (Calbiochem #475904) mounting medium, sealed, and imaged at 10, 20, or 40X on an Olympus BX51 microscope (Center Valley, PA, USA) equipped with a CoolSNAP E52 camera (Photometrix, Tucson, AZ, USA) under the control of Image Pro 6.2 software (Media Cybernetics, Rockville, MD, USA) or with a Zeiss Axiovert 200M equipped with an Axiocam MRm). In experiments where MCs and ECs were co-cultured, ECs were pre-labeled with 5 μM SP-DiOC$_{18}$(3) (Life Technologies Cat.# D-7778) in DMSO or PKH26 (Sigma-Aldrich, St. Louis, MO, USA) as per the manufacturer's instructions. Ratios of the average fluorescence intensity value (per MC nucleus) were obtained from paired experiments containing MCs cultured alone or co-cultured with ECs. For convenience the ratios were expressed as percent of control values.

Dye coupling: *L*MC-43 and *L*Cx43-S3A cells in solo culture were injected with a mixture of dyes including NBD-M-TMA[46], a small, cationic dye that is gap junction permeable, and rhodamine-dextran, a large, gap junction impermeable dye. Transfer of NBD-M-TMA, but not rhodamine-dextran, to at least one neighboring cell indicated the presence of functional gap junctions between cells and was scored as positive for coupling. Where heterocellular coupling (EC to MC) was examined, ECs were prelabeled with PKH26 (Sigma- Aldrich) to distinguish them

from unlabeled MCs, and rhodamine-dextran was not included in the injected dye mixture. The presence of functional gap junctions between ECs and either MC40, MC43-S368A, MC43-S255A or MC43-S2A was tested by injecting Lucifer yellow and determining whether any neighboring cells were positive for dye after 2 minutes.

Western Blot: MCs plated at the densities outlined above, were cultured with or without TGF-β1(1ng/ml)-Calibiochem) or co-cultured with ECs for 48h in LG-DMEM containing 2% FBS. After this 48 h treatment period, total cell protein was isolated for determination of SM α-actin and calponin expression. Cells were lysed with Laemlli buffer and protein concentration in the collected lysate was determined using the BCA assay. Using 12% PAGE gels, 10 µg of total protein was loaded in each lane solo MCs cultures and 20 µg/lane for 1:1 EC:MC co-cultures to allow direct comparison of solo and co-culture data. (As previously published[4] we operated under the assumption that half of the protein from co-culture samples was contributed by MCs so quantitative analysis of 2X protein allows direct comparison with MC-only data). Proteins were separated in an electric field, transferred to a nitrocellulose membrane and stained for SM specific α-actin (Dako, 1:2500) and calponin (1:2000) (Millipore, Billerica, MA, USA). Chemiluminescent techniques were employed to quantify protein expression (BioRad Chemidoc XRS, Hercules, CA, USA)). All experiments were performed at least three times.

Statistical Analysis: Single sample t-scores were used to determine whether SM α-actin expression was increased by addressing whether the ratio of treated to control was different from 1. In all cases significance was set at the $p \leq 0.05$

Supplemental Figures:

Figure 1: Cx43 expression and gap junction function in $MC43^{-/-}$ and *L*MC43 cells

Consistent with previously published data[4,30] $MC43^{-/-}$ cultures fixed and immunostained for Cx43 (white) revealed no detectible Cx43 signal (co-stained with Hoescht dye to reveal nuclei – blue). (B-D) $MC43^{-/-}$ cells are not dye-coupled despite the presence of active Cx45 gap junction channels[4,30]. In this example, a single cell (asterisk in B) was injected with a mixture of gap junction permeable NBD-M-TMA (NBD) and gap junction impermeable rhodamine-dextran (Rhodex). Neither dye was detected in any neighboring cells after 5 minutes (compare D & E to C; data representative of 10 injections). In contrast, in *L*MC43 cultures (F) Cx43 (white) was readily detected intracellularly and at appositional membranes as punctate labeling (F-inset), indicative of gap junction formation. (G-H) The *L*MC43 cells were consistently dye-coupled, with intercellular diffusion of the gap junction permeable NBD-M-TMA from the injected cell (asterisk in G) to a neighboring cell (compare panel J to H) without simultaneous intercellular diffusion of the gap junction impermeable rhodamine-dextran (compare panel I to J). The scale bars represent 100 µm.

Figure 2: MAPK mutants of Cx43 express well and form functional gap junctions with ECs.

Cx43-S255A (A) and Cx43-S279,282A (B) protein is readily detected in total protein isolated from MC43-S255A and MC43-S2A (clone 3 cells), as well as in wild-type expressing MCs (Re43), but not in MC43$^{-/-}$. Both mutant proteins are also readily detected by immunocytochemistry (pink in Panel C). (D,E) For both mutants, Lucifer yellow diffuses from an injected EC (asterisk, PKH26 labeled) to neighboring MC (no PKH26 labeling). The scale bar represents 100μm.

Figure 3: MC43-S255A in co-culture with ECs do not upregulate mural cell markers but respond to TGF-β

Immunocytochemistry (A,B, n=3) and western blot (C, n=3) experiments revealed no up-regulation of SMA by MC43-S255A when co-cultured with ECs. However, some increase in SMA following stimulation with exogenous TGF-β (D,E; n=3 and F, n=3).

Figure 4: MC43-S2A (clone # 3) in co-culture with ECs do not upregulate mural cell markers but respond to TGF-β

Immunocytochemistry (A,B, n=1) and western blot (C, n>3) experiments revealed no up-regulation of SMA or other mural cell markers by MC43-S2A cells when co-cultured with ECs, but a limited increase in SMA was observed following stimulation with exogenous TGF-β (D,E; n=3)

Figure 5: MC-S368A form functional gap junctions with ECs.

MC-S368A are capable of communicating with ECs. In this representative experiment (n>30), lucifer yellow diffuses from an injected EC (asterisk, PKH26 labeled) to neighboring MC (no PKH26 labeling). The scale bar represents 100μm.

Figure 6: Cx40 is expressed well in MC40 cells and these cells form functional gap junctions with ECs.

We previously reported that Cx43$^{-/-}$ MCs do not express Cx43, Cx40, or Cx37, and only very small amounts of Cx45, which are not sufficient to support EC-MC dye coupling[4]. (A) RT-PCR amplicons from MC43$^{-/-}$ cells transfected with Cx40 (MC40) show that Cx40 mRNA is transcribed after transfection but not before (vehicle lanes). (B) Western blot experiments with whole cell lysates from MC43$^{-/-}$ (vehicle lane) and MC40 cells reveal that Cx40 protein is translated in MC40 cells but not parental MC43$^{-/-}$ cells. (C) Immunostaining of fixed MC40 cells with anti Cx40 antibody reveal that the protein is properly localized, with punctate labeling at appositional membranes. (D) MC40 cells form functional gap junctions with ECs (n>30). In this representative experiment, Lucifer yellow injected into an MC40 (marked with asterisk) cell diffuses neighboring ECs (marked with PKH 26 (and white stars)) and neighboring MCs (black star).

Fig. 1

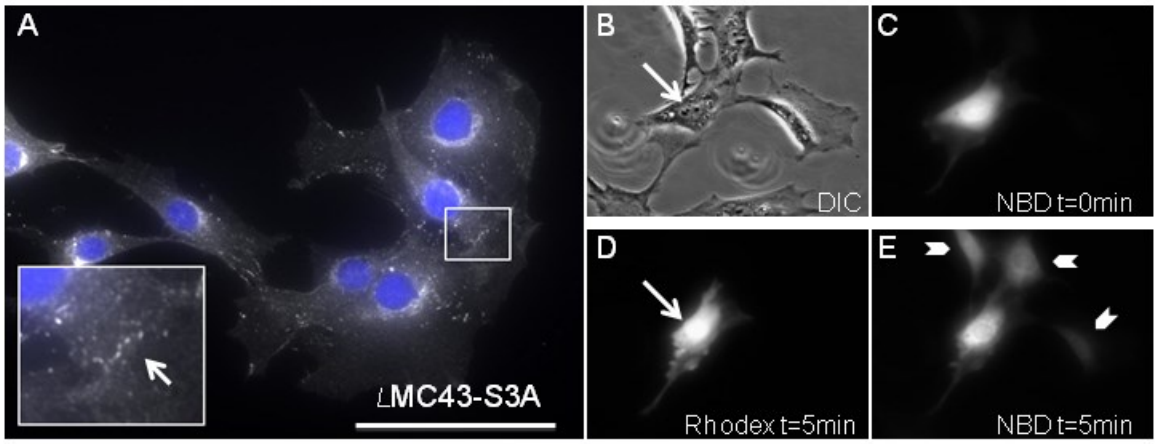


Fig. 2

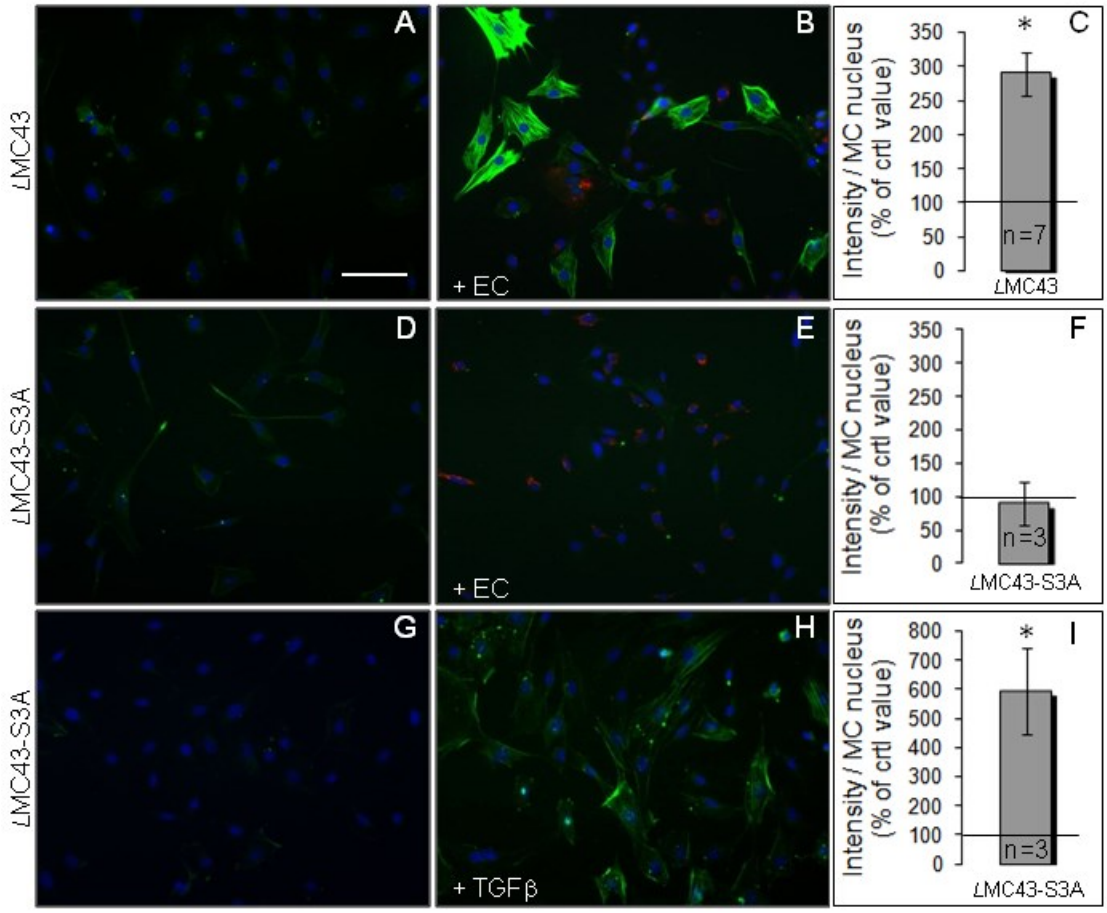

ΔMC43
ΔMC43-S3A
ΔMC43-S3A
A
B
C
D
E
F
G
H
I
+ EC
+ EC
+ TGFβ
Intensity / MC nucleus (% of crtl value)
n = 7
n = 3
n = 3
ΔMC43
ΔMC43-S3A
ΔMC43-S3A
*
*

Fig. 3

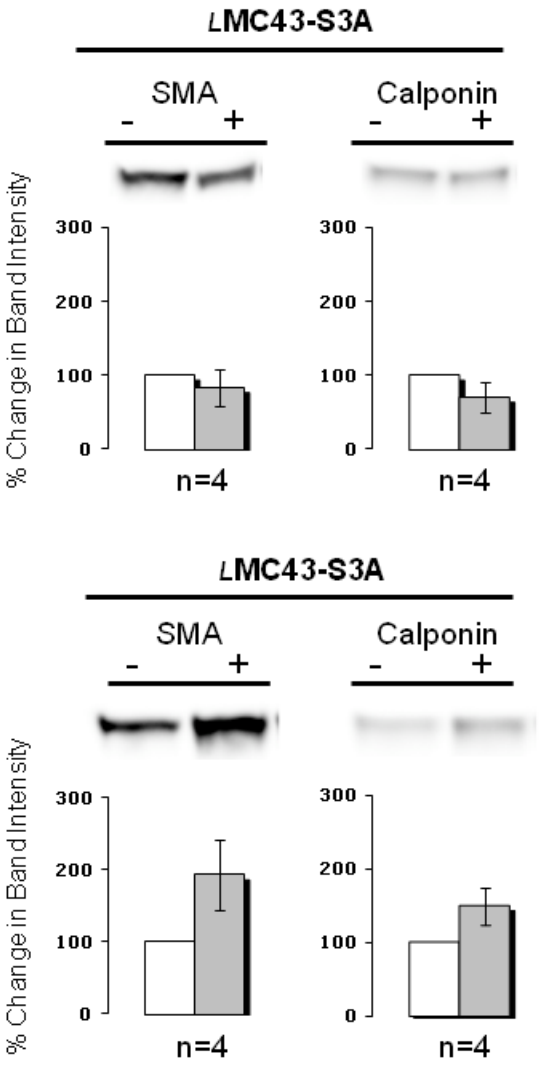

LMC43-S3A
SMA
Calponin
- +
- +
% Change in Band Intensity
300
200
100
0
n=4
n=4
LMC43-S3A
SMA
Calponin
- +
- +
% Change in Band Intensity
300
200
100
0
n=4
n=4

Fig. 4

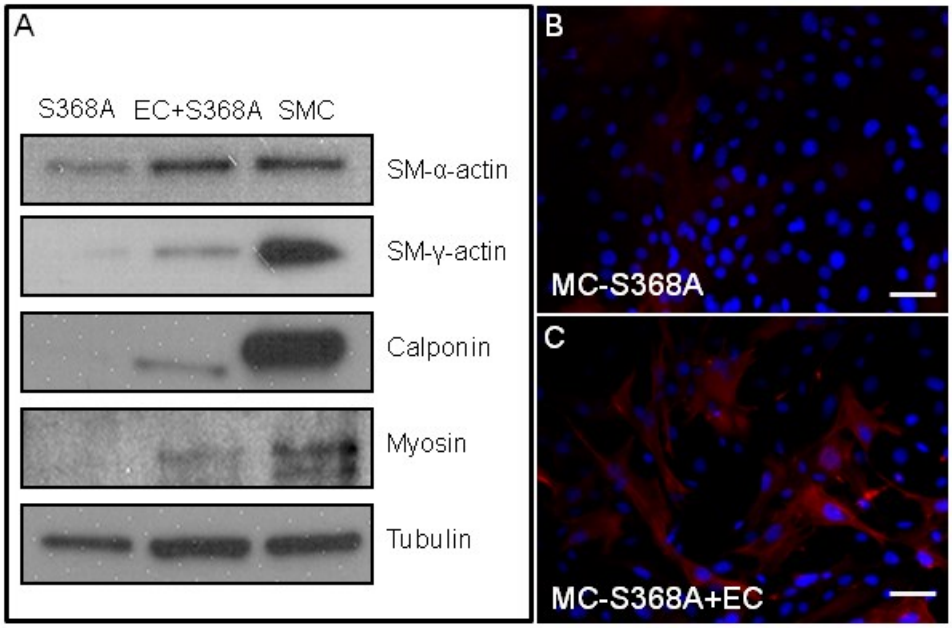
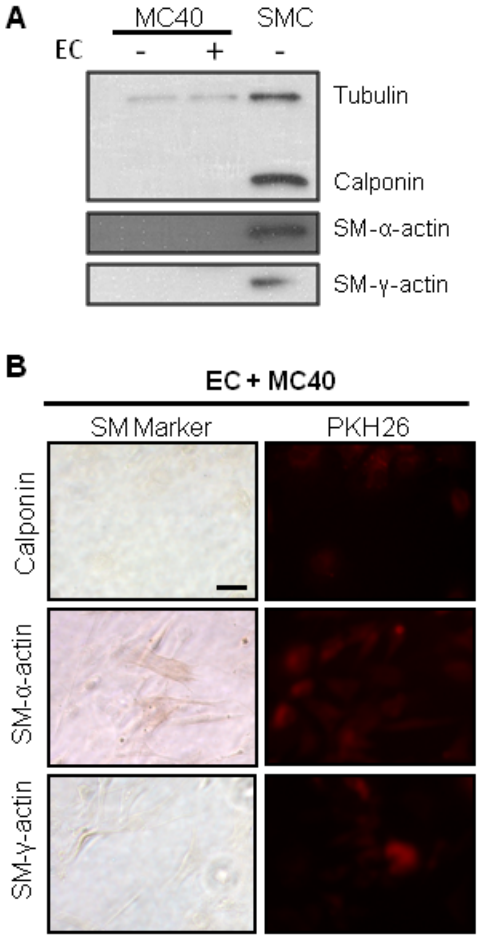

A
S368A
EC+S368A
SMC
SM-α-actin
SM-γ-actin
Calponin
Myosin
Tubulin
B
MC-S368A
C
MC-S368A+EC

Fig. 5


A
MC40
SMC
EC
-
+
-
Tubulin
Calponin
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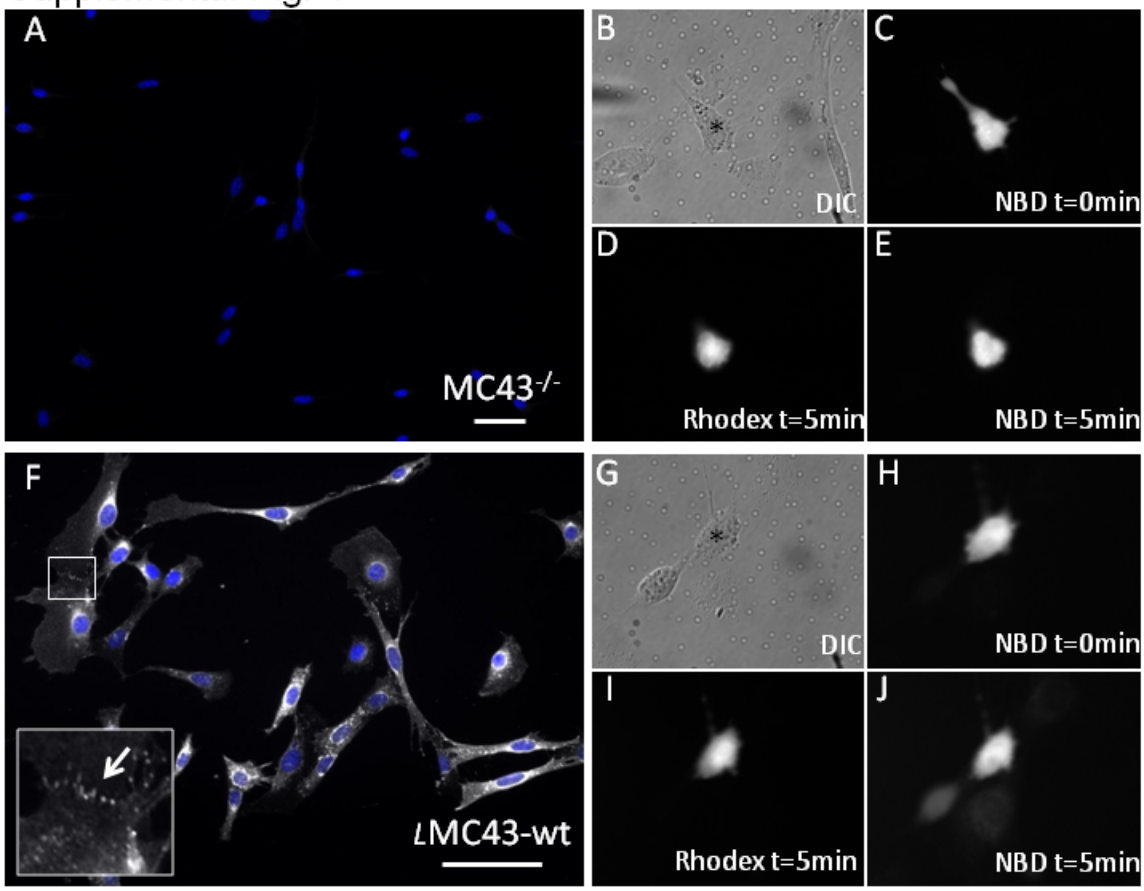
B
EC + MC40
SM Marker
PKH26
Calponin
SM-α-actin
SM-γ-actin

Supplemental Fig. 1



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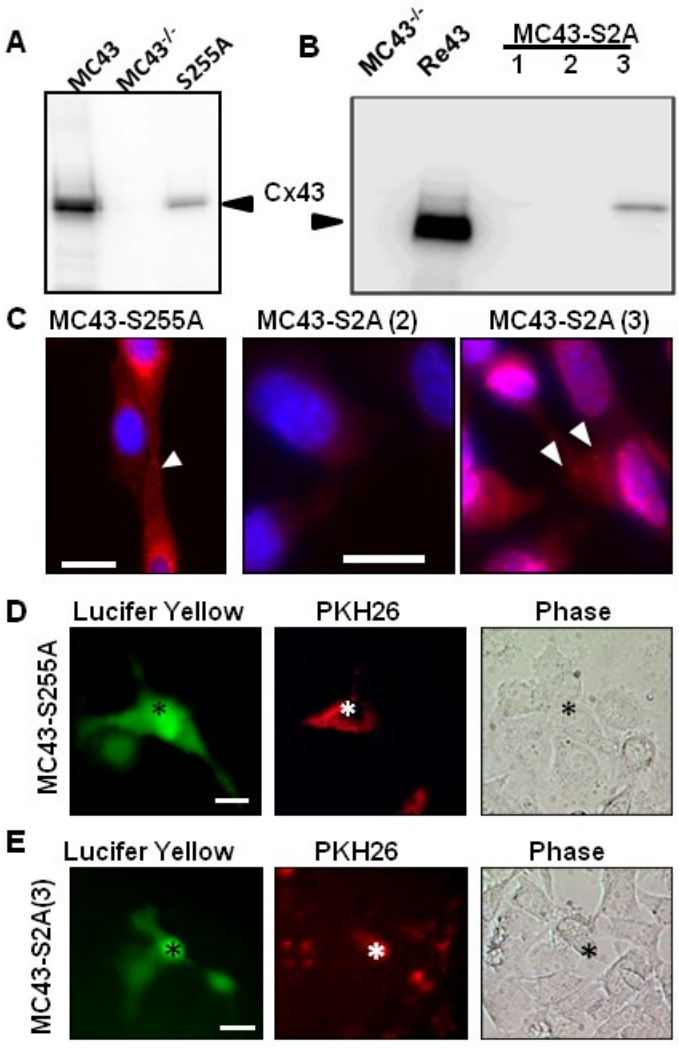

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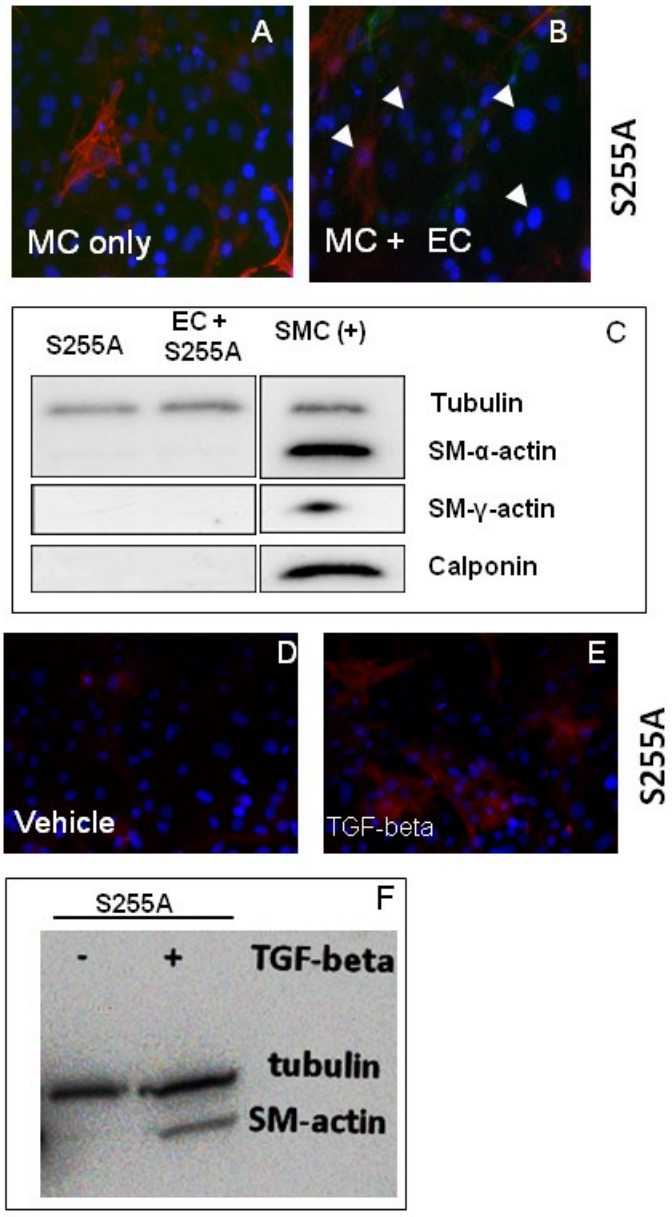
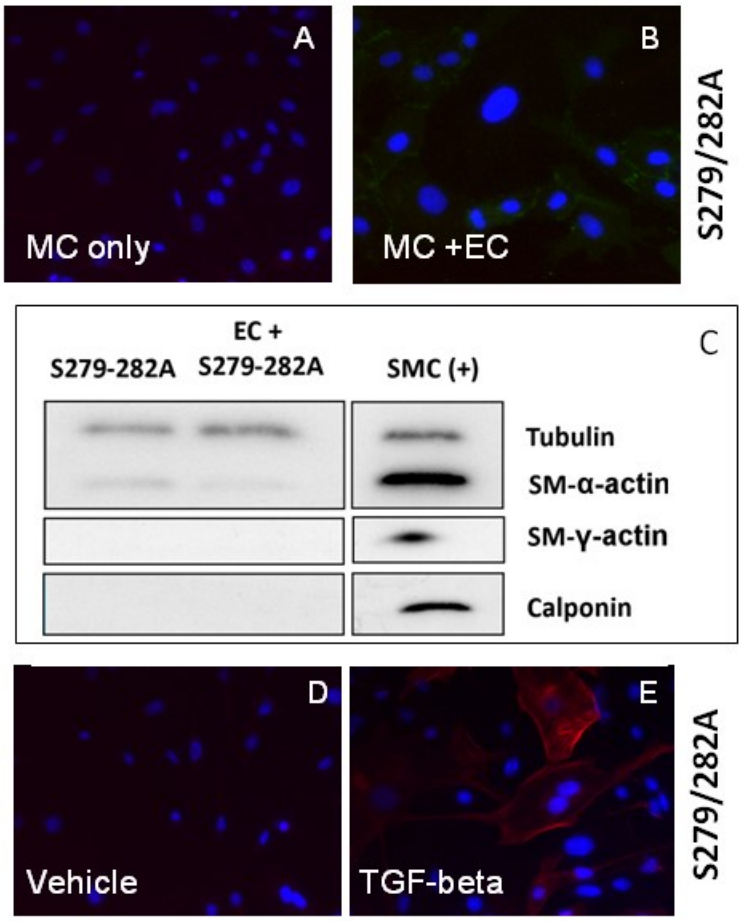


Supplemental Fig. 4



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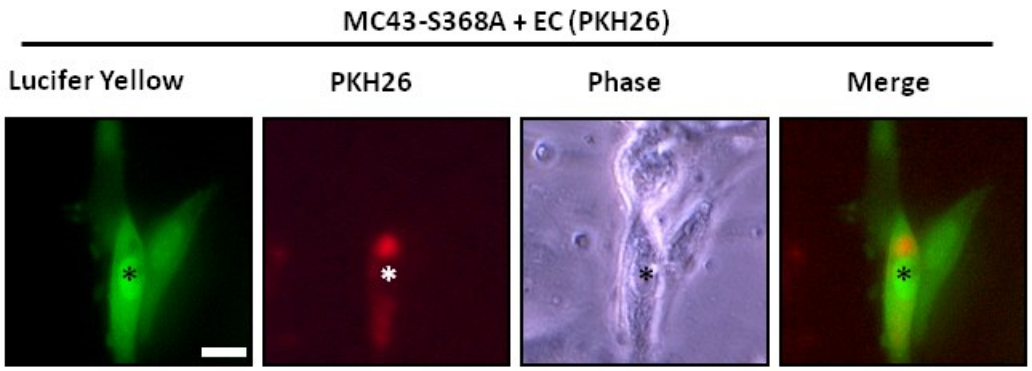
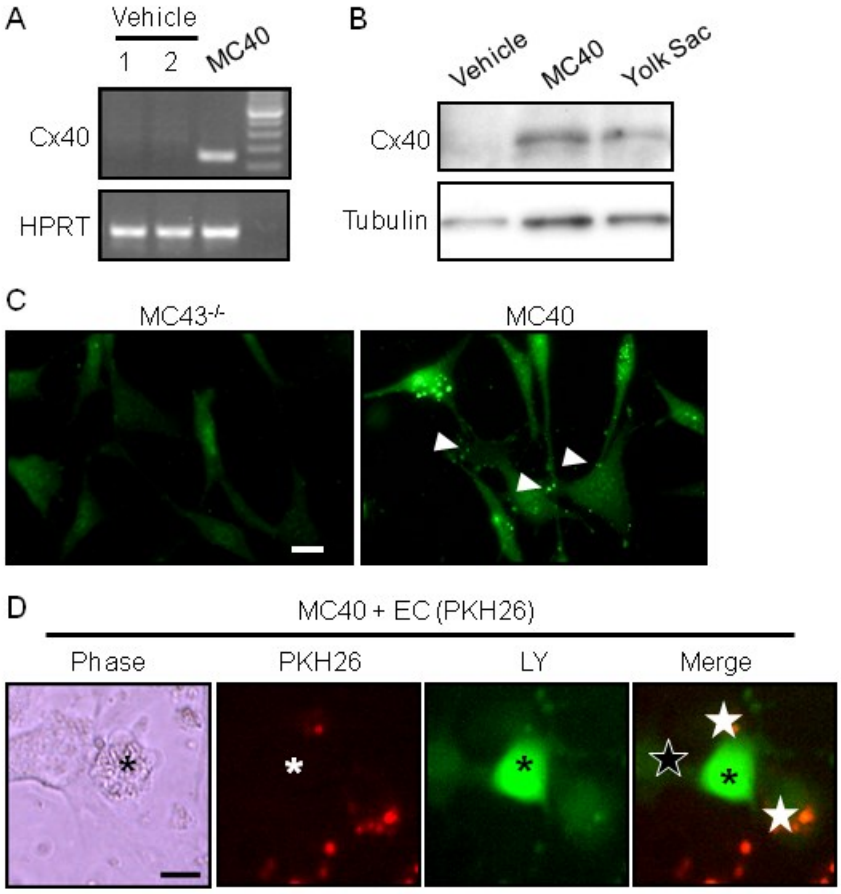


Supplemental Fig. 6



APPENDIX C
(Prepared for Publication at *BBA-Biomembranes*)

INCREASED $[CA^{2+}]_I$ AND CX43-COMPRISED GAP JUNCTIONS SUPPORT ENDOTHELIAL CELL-INDUCED MURAL CELL DIFFERENTIATION BY DISTINCT MECHANISMS.

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Short Title: Endothelial-induced mural cell differentiation

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Word count body:
Word count abstract: 182
Total number of figures and tables: 6

Abstract

Objective – Gap junction mediated intercellular signaling is required for endothelial cell (EC)-induced, TGF-β-dependent, mesenchymal cell (MC) differentiation to a mural cell phenotype. Here we determined whether an increase in EC intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, could replace Cx43-mediated signaling in EC-induced MC differentiation.

Methods and Results – Ionomycin, a Ca^{2+} ionophore, was used to increase $[Ca^{2+}]_i$ in ECs, MCs deficient ($MC^{-/-}$s) or not (Re43) for Cx43, or EC-MC co-cultures. Expression of mural cell markers was quantified in ionomycin treated cells and in naïve $MC^{-/-}$s treated with medium conditioned by ionomycin treated cells. Ionomycin increased expression of mural cell markers in $MC^{-/-}$ only when they were in co-culture with ECs or when exposed to medium conditioned by ionomycin-treated ECs or EC-$MC^{-/-}$ co-cultures. Ionomycin-EC dependent differentiation of MCs was unaffected by inhibitors of TGF-β or matrix metalloproteinase activity, but required activin-like kinase (ALK) receptor (4, 5, or 7) activation.

Conclusion – Our data show that ionomycin-induced elevation of $[Ca^{2+}]_i$ in ECs is not sufficient to replace functional EC-MC, Cx43-comprised gap junctions in EC-induced, TGF-β-dependent, MC differentiation. $[Ca^{2+}]_i$ elevation in ECs nevertheless supported a modest differentiation of MCs.

Key Words: endothelial cell, mesenchymal cell, ALK receptor, mural cell differentiation

1. Introduction

The formation of new blood vessels by vasculogenic and angiogenic mechanisms involves endothelial cell (EC) secretion of platelet-derived growth factor-B (PDGF-B), which acts as a chemoattractant and mitogen for mural precursor cells[16,17,24]. PDGF-B stimulates these cells (mesenchymal cells: MCs) to migrate towards and ultimately contact the ECs. These heterocellular contacts, which include gap junctions (GJs), support transmission of a signal(s) between MC and EC that, in turn, leads to release of soluble factors (SFs: SF1, SF2 etc.) by the ECs. These SFs activate transforming growth factor-beta (TGF-β; and possibly other ligands), which induce MC differentiation toward a mural cell phenotype by binding to TGF- β type II receptors (TβRII) that recruit and phosphorylate TGF- β type I receptors (e.g., activin-like kinase 5 (ALK5)) thereby enabling Smad2/3 phosphorylation (pSmad2, pSmad3, pSmad2/3)[5,33]. pSmad2/3 then complexes with co-Smad4 and translocates to the nucleus where it interacts with transcription factors (e.g. serum response factor, SRF) to regulate transcription of target genes that support mural cell differentiation[5,33]. A key aspect of this scenario that remains uncertain is the identity of the signal(s) transmitted by the GJ between MC and EC that leads to release by the EC of the TGF- β activating factor(s) and subsequent response in the MC to TβRII activation.

We demonstrated previously[15] that connexin 43 (Cx43) deficient MCs ($MC^{-/-}$s) fail to differentiate when co-cultured with ECs despite retaining the capacity for differentiation in response to exogenously applied TGF-β. Stable re-introduction of Cx43 into $MC^{-/-}$s (Re43) restored their capacity for intercellular communication and their potential for EC-induced, TGF-β dependent, MC differentiation towards a mural cell fate, suggesting that restoration of intercellular communication supported transmission of a signal to the EC that was necessary for stimulating release of a TGF-β activating factor. The signal(s) communicated between MC and EC remains unidentified, but clearly must support secretion of the TGF-β activating factor.

Ca^{2+} is typically required for stimulus-secretion coupling[14,26,31] and PDGF-B receptor activation elevates $[Ca^{2+}]_i$ by activating phospholipase C gamma (PLC΄Ύ), inositol trisphosphate (IP_3) formation and subsequent IP_3-mediated release of Ca^{2+} from intracellular stores[4,22]. Since Cx43-comprised GJs are permeated by both Ca^{2+} [27,34]and IP_3, [3,6] we hypothesized that Ca^{2+} might be the GJ-transmitted signal between MC and EC that results in EC-release of a TGF- β activating factor. Thus, the goal of the current study was to determine whether elevation of $[Ca^{2+}]_i$ in ECs is sufficient to replace Cx43 in GJ-dependent, EC-induced, TGF-β-mediated, mural cell differentiation. Because net intercellular flux of Ca^{2+} (and other molecules) occurs by diffusion when an electrochemical gradient occurs between GJ-coupled cells, we used the Ca^{2+} ionophore ionomycin, which has no direct effect on endogenous Ca^{2+} flux and storage mechanisms, to simulate the expected effect of GJ-mediated elevation of $[Ca^{2+}]_i$

in ECs. We demonstrate that elevation of $[Ca^{2+}]_i$ in this manner did not substitute for GJs in EC-induced, TGF- β dependent, mural cell differentiation, suggesting that Ca^{2+} is not the GJ-transmitted signal that stimulates differentiation and, indeed, may not be sufficient for this TGF-β-dependent differentiation event. However, ionomycin did initiate TGF-β-independent, but ALK 4, 5 and/or 7-dependent differentiation of MCs towards a mural cell phenotype. The identity of the SFs released in the context of either GJ-dependent or ionomycin-dependent, EC-induced mural cell differentiation remains unknown, although matrix metalloproteinases (MMPs) do not appear to be involved.

2. Methods

2.1. Cells: ECs were isolated from the bovine aorta as outlined previously,[8,17] grown in low glucose Dulbecco's Minimal Essential Medium (LG-DMEM) with 10% fetal bovine serum (FBS; Gemcell, Gemini Bio-Products, Sacramento, CA) in a humidified incubator (37°C and 5% CO_2) and studied between passages 15 and 20. $MC^{-/-}$, originally isolated from day 15.5 embryonic Cx43 knockout mice,[25] and Re43 cells ($MC^{-/-}$ cells transfected or infected to constitutively express Cx43)[15,25] were grown under similar conditions. Cultures of a single cell type (EC, Re43 or $MC^{-/-}$) were seeded at $6x10^5$ cells per 100 mm plate, whereas co-cultures were seeded with $3x10^5$ ECs and $3x10^5$ $MC^{-/-}$ or Re43 one day prior to use.

2.2. $[Ca^{2+}]_i$ measurement: Cells, alone or in co-culture, were grown on glass cover slips at the density outlined above. Prior to co-culture with either $MC^{-/-}$ or Re43 cells, ECs were labeled with 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO; 0.5 μM) to facilitate distinguishing these cell types in co-culture. After 24h the cells were loaded with fura-2-AM (5 μM at 37°C for 30 min). Fura-2 fluorescence was recorded at 505 nm using an Olympus IX70 microscope and ICCD camera (Photon Technologies Incorporated (PTI); Monmouth Junction, New Jersey) after alternate excitation at 340 and 380 nm by a 75 W Xenon lamp linked to a Delta Ram V illuminator (PTI) and a gel optic line (measurements conducted at room temperature). The imaging system was under the software control (ImageMaster, PTI) of an IBM clone computer. Ratios were obtained every 0.6 sec over a 5 min period. To avoid possible variability stemming from day-to-day differences in fura-2-AM loading, room temperature, or equipment calibration, all comparisons were made in a paired fashion between data sets obtained on the same day and using the same calibration data. (Fura-2 was calibrated against 0 and 1mM Ca^{2+}; 340:380 fluorescence ratios were converted to $[Ca^{2+}]$ as described by Grynkiewicz[11].) As phenol red and extracellular proteins in complete medium can affect fura-2 measurements, cells were treated with serum and phenol red free medium (LG-DMEM) containing 0.5 μM ionomycin (from 1 mM stock ionomycin in DMSO) or the vehicle control (0.05% DMSO).

2.3. Western Blot: Cells were lysed with Laemlli buffer, total protein harvested, and protein content determined using the bicinchoninic acid (BCA) assay. Polyacrylamide gels (12%) were loaded with 4 μg total protein/lane for $MC^{-/-}$ or 8

μg/lane for 1:1 $MC^{-/-}$-EC co-cultures[15]. In experiments aimed at detecting pSmad2 and pSmad3 in $MC^{-/-}$s, 25 μg and 10 μg total protein, respectively, were loaded on gels. Proteins were separated in an electric field, transferred to a nitrocellulose membrane and stained for smooth muscle α actin (SMA, (Dako; Carpinteria, CA)), calponin (Dako; Carpinteria, CA), pSmad2 (Cell Signaling Technology; Beverly, MA) or pSmad3 (Epitomics, Inc; Burlingame, CA). Chemiluminescent techniques were employed to quantify protein expression (Kodak; Rochester, NY). Results were quantified by densitometry, and the data from multiple blots evaluated for statistically significant differences.

2.4. RT-PCR and Southern Blot: The Cells-to-cDNA™ II kit (Ambion, cat# AM1722M) was used to combine cell lysate and reverse transcription reactions for $1x10^4$ cells into a single step. 3T3 cells were used as the positive control for ALK4; Min6 cells were used as the positive control for ALK7. The resulting reverse transcription products (2 μl) were used in polymerase chain reaction to amplify ALK4 and ALK7 cDNA with specific primers. ALK4 and ALK7 primers were designed using Primer-BLAST (NCBI website). ALK4 primers were (5' to 3'): F1-GTGCGTGCACCAGCTGCCTA, R1- GGGTGCGCAGGCTCCTTGAG and F2-AGCCTGGCTGTGGGTGGTGA, R2- GGCCGAGGGTGCAGGGAGTA. ALK7 primers were (5' to 3'): F1-TCACCGTGCCGGTTTGCCTC, R1-TCGTGCTTCACAGCCAGCCC; F2-AGGTCCTGCCCTCGGGGATT, R2-GCCAGCTCTCCCTTCGCACG; and F3-GTGTGATTCTTCAAACTTCAC, R3-GTATGTCTATAGTGTTCATGA. Primers were used at a concentration of 20 pmol per reaction. The PCR protocol for ALK7 primers was: 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, followed by 72°C for 5min. The PCR protocol for ALK4 primers was identical except the annealing temperature was 55 °C. PCR products were separated by electrophoresis (100 volts for 40 min) in 0.7% agarose gels containing ethidium bromide.

2.5. Experimental Paradigms:

Direct treatment protocol:

Cells, plated at the densities outlined above, were allowed to settle overnight in medium containing 10% FBS. They were then exposed for 48h to LG-DMEM containing 2% FBS and 0.05% DMSO with or without ionomycin (0.5 μM). The protein content of whole-cell lysate (WCL) was determined and mural cell marker expression in WCL evaluated by western blotting. Changes in expression are reported as the ratio of ionomycin treated to vehicle (DMSO) treated control.

Conditioned Medium Protocol: Cells were plated and treated as described above for the direct treatment protocol. After the 48h treatment time, the medium bathing the cells (conditioned medium) was collected and applied to naïve $MC^{-/-}$s for either 60 minutes or an additional 48h. WCL was evaluated for SMA and calponin content (48h treatment) or pSmad2/3 content (60 minutes).

To evaluate the potential contribution of specific signaling cascade elements in observed differentiation responses, additional treatment groups were included in

the appropriate paradigms. To determine whether MMPs might be responsible for formation of activated SFs, the MMP inhibitor GM-6001 (10 μM; a concentration previously shown to inhibit MMP activity [32]) was added during ionomycin treatment and generation of conditioned medium. To determine whether TGF-β was the factor activated as a consequence of ionomycin treatment, TGF-β neutralizing antibody (10 μg/ml; R & D Systems) was added to the conditioned medium prior to its application to $MC^{-/-}$s. To determine the involvement ALK4, 5, and 7 receptor-dependent activation of Smad2/3, SB 431542 (10 μM, a concentration previously shown to inhibit pSmad activation by these receptors [20]) was added to the conditioned medium prior to exposing $MC^{-/-}$s to this medium. Finally, the dose-dependence of $MC^{-/-}$ response to activin A and activin B (0 - 500 ng/ml in 2% FBS LG-DMEM for 48 hrs) was determined.

2.6. Statistical Analysis: Students t-test was used to compare the means of two samples. Single sample t-scores were employed to determine whether SMA expression was increased by addressing whether the ratio of treated to (vehicle) control was different from 1. Significance was set at the $p \leq 0.05$ level.

3. Results

We first determined $[Ca^{2+}]_i$ in ECs (n=39) and MCs (n=11 for Re43 and 33 for $MC^{-/-}$) at baseline and in response to direct ionomycin treatment (**Figure 1A**). Comparison of basal $[Ca^{2+}]_i$ in a paired fashion across cell types revealed that basal $[Ca^{2+}]_i$ in Re43 and $MC^{-/-}$ was significantly greater than in ECs (EC vs. Re43: 71±8 nM, n=11 vs. 20±2 nM, n=15, $p<0.001$; $MC^{-/-}$ vs. EC: 84±10 nM, n=24 vs. 26±2 nM, n=24, $p<0.001$), suggesting that at least a temporary intercellular $[Ca^{2+}]_i$ gradient could occur upon GJ formation between Re43 and EC cells. Ionomycin application caused $[Ca^{2+}]_i$ to increase in all cell types (**Figure 1A**), but peak concentration was lower in ECs, 268 ± 19 nM, than either Re43 or $MC^{-/-}$, 673 ± 38 and 661 ± 37 nM, respectively. After ~5 minutes in the continued presence of ionomycin, $[Ca^{2+}]_i$ remained elevated in all cell types, but to a greater extent in $MC^{-/-}$ than in Re43 cells. 24h after introduction of ionomycin $[Ca^{2+}]_i$ in ECs had returned to baseline levels (**Figure 1B**; 31±3 nM, n=11 vs. after 24h in ionomycin: 35±4 nM, $p>0.3$). We next examined basal $[Ca^{2+}]_i$ in ECs co-cultured or not with Re43 or $MC^{-/-}$ cells. As shown in **Figure 1B**, no differences in basal EC $[Ca^{2+}]_i$ were observed as a consequence of co-culture with either Re43 cells (EC: 21±1.4nM, n=29 vs. EC:co-Re43: 17±4nM, n=5; $p>0.35$) or $MC^{-/-}$ cells (EC: 26±2 nM, n=25 vs. EC:co-$MC^{-/-}$: 24±3, n=14; $p>0.55$). These data suggest that GJ formation between EC and MC did not result in a sustained increase in EC basal $[Ca^{2+}]_i$; however, a transient transjunctional $[Ca^{2+}]$ gradient likely occurred early in the ionomycin treatment period, given the difference in peak $[Ca^{2+}]_i$ response of these cell types.

To determine whether direct elevation of $[Ca^{2+}]_i$ in $MC^{-/-}$ or $MC^{-/-}$:EC co-cultures was sufficient to induce $MC^{-/-}$ differentiation toward a mural cell phenotype, we added ionomycin directly to the culture medium and assayed for SMA expression 48h later (**Figure 2A**-Direct Treatment Protocol). As shown previously[15] and here, direct treatment with TGF-β (1 ng/ml) for 48h induced increased SMA expression in $MC^{-/-}$s ($p<0.05$: treated/control > 100%; **Figure 2B and 2D**). Direct

treatment of $MC^{-/-}$s with ionomycin (0.5 μM; 48h) induced no significant change in SMA expression. SMA was not detected in ECs whether they were treated with ionomycin or not. When $MC^{-/-}$s were co-cultured with ECs, ionomycin treatment induced a significant (compared to vehicle treated controls) increase in SMA expression ($p<0.05$; **Figure 2B and 2D**). Similar results were observed when the expression of other mural cell markers, including calponin, was assessed (data not shown).

Based on these results, we hypothesized that elevating $[Ca^{2+}]_i$ in ECs co-cultured with $MC^{-/-}$s might trigger the ECs to condition their medium (to release a SF) such that the medium would stimulate $MC^{-/-}$ differentiation towards a mural cell phenotype. If this hypothesis were correct, then medium conditioned by EC:MC co-cultures (would contain this SF), when applied to naïve $MC^{-/-}$s, would stimulate the $MC^{-/-}$s to differentiate. To evaluate this possibility, $MC^{-/-}$s or EC:$MC^{-/-}$ co-cultures were treated with ionomycin or DMSO vehicle for 48h and the medium harvested for subsequent treatment of naïve $MC^{-/-}$s (**Figure 2A – Conditioned Medium Protocol**). The conditioned medium from ionomycin treated EC:$MC^{-/-}$ co-cultures increased (relative to vehicle treated controls) SMA expression ($p<0.05$) when applied to naïve $MC^{-/-}$s (**Figures 2C and 2E**). In contrast, medium conditioned only by $MC^{-/-}$s treated with ionomycin had no effect on expression of SMA by naïve $MC^{-/-}$ (**Figures 2C and 2E**). To ascertain whether the $MC^{-/-}$s were required for release of this SF, ECs alone were treated with ionomycin (or vehicle) for 48h and the resulting conditioned medium applied to naïve $MC^{-/-}$s for an additional 48h. The ionomycin-EC conditioned medium, like the medium conditioned by EC: $MC^{-/-}$ co-cultures, induced increased ($p<0.05$) expression of SMA when applied to naïve $MC^{-/-}$s (**Figures 2C and 2E**). These data indicate that elevation of $[Ca^{2+}]_i$ in ECs, co-cultured or not with $MC^{-/-}$s, is sufficient to condition medium (release a SF(s)) such that it stimulates upregulation of SMA when applied to naïve MCs.

To determine whether ionomycin-ECs conditioned medium stimulated upregulation of SMA in a TGF-β-dependent manner, we added a TGF-β1,2,3 neutralizing antibody (10 μg/ml) to the conditioned medium before applying that medium to naïve $MC^{-/-}$s (**Figure 2A conditioned medium protocol**). At concentrations able to block TGF-β mediated differentiation (**Figure 3A** and Hirschi et al[15]), the neutralizing antibody failed to block the increase in SMA expression induced by the conditioned medium, indicating that TGF-β was not responsible for the observed ionomycin-EC conditioned medium-induced differentiation. However, the data show that elevation of $[Ca^{2+}]_i$ in ECs initiated an alternative signaling sequence that also conditioned the medium such that when applied to naïve $MC^{-/-}$s mural cell differentiation was stimulated.

Members of the TGF- β superfamily of ligands are secreted in latent or active forms that bind to the extracellular matrix through protein-protein or protein-glycosaminoglycan (GAG) interactions; such interactions serve to localize these and other growth factors to specific tissue regions[23]. Bound growth factors can be released from the matrix and from latency binding proteins by endopeptidases such as the MMPs,[28] thereby increasing their bioavailability.[32] To determine

whether ionomycin-EC conditioning of medium depended on MMP activity, we next determined whether the presence during conditioning of a broad spectrum MMP inhibitor (GM-6001; targeting MMPs 1, 2, 3, 8 & 9) blocked the increase in SMA expression typically induced by ionomycin:EC conditioned medium. As shown in **Figure 3B**, ionomycin-EC conditioned medium evoked an increase in SMA expression by naïve $MC^{-/-}$s whether or not MMPs were inhibited, suggesting that MMPs were not involved in ionomycin-EC medium conditioning.

Mural cell differentiation typically involves activation of R-Smads via ALK 4, 5 or 7 receptors (TGF-β sub-family Type I receptors) upon their activation by ligand-bound TβRII.[5,33] If this pathway were involved in ionomycin-EC-induced, $MC^{-/-}$ differentiation, then inhibiting Smad2/3 activation with SB-431542[18,20] would be expected to block the increase in SMA expression in $MC^{-/-}$s. To test this possibility, 10 μM SB-431542 was added to ionomycin-EC conditioned medium prior to its application to naïve $MC^{-/-}$s; inhibition of ALK4, 5, 7-receptor kinase activity completely blocked the increase in SMA expression ($p<0.05$, **Figure 3C**) induced by the conditioned medium, demonstrating the central importance of Smad2/3 activation (and possibly some Smad-independent downstream signaling events) to ionomycin-EC-induced $MC^{-/-}$ differentiation.

To further elucidate the role of pSmad2 and pSmad3 in ionomycin-EC-induced $MC^{-/-}$ differentiation, we compared the levels of pSmad2 and 3 before and after treatment of $MC^{-/-}$s with either TGF-β (1ng/ml) or medium conditioned by ECs (treated or not with ionomycin). TGF-β resulted in a significant (350 - 450%) increase in pSmad2 and 3 (**Figure 4A**). Ionomycin-EC conditioned medium resulted in a statistically significant, but smaller increase in pSmad3 (relative to vehicle treated controls) and no change in pSmad2 level (**Figure 4B**). Smad signaling is activated by ALK4, 5 and 7 type 1 receptors when these receptors complex with, and are activated by, a Type 2 receptor. Since the conditioned medium from ionomycin treated ECs induced $MC^{-/-}$ differentiation via a TGF- β1,2 or 3- independent mechanism (and therefore likely independent of ALK5 Type 1 receptor activation), we next determined whether ALK4 or 7 receptor transcripts were expressed by the $MC^{-/-}$s. **Figures 4C&D** show that reverse transcription dependent PCR amplicons for both receptors can be generated from the $MC^{-/-}$s.

Finally, to gauge whether activation of either the ALK4 or ALK7 receptors produced upregulation of SMA in $MC^{-/-}$ comparable to that induced by ionomycin-EC conditioned medium, we treated $MC^{-/-}$s with increasing doses of either Activin A or Activin B (using the Direct Treatment Protocol, **Figure 2A**). Activin B induced a dose-dependent increase ($p<0.05$) in SMA expression (**Figure 5**) by $MC^{-/-}$s, whereas low concentrations of Activin A produced a small, but insignificant, inhibition of SMA expression and at higher concentrations had no effect. Since ALK4 receptors are activated by both Activins whereas ALK7 receptors are far better activated by Activin B or Activin AB than Activin A[30], we suspect that ALK7 is more likely than ALK4 or 5 to be the type 1 receptor mediating the ionomycin-EC induced increase in SMA expression by $MC^{-/-}$s.

4. Discussion

Previous studies demonstrated that EC-induced mural cell differentiation is mediated by TGF-β, which is activated by a SF (**SF1 in Fig 6**) secreted by endothelial cells (thereby conditioning the bathing medium). This sequence of signaling events depended critically on the ability of ECs and MCs to communicate via Cx43 (or Cx45) comprised GJs[15]. However, the identity of the signal(s) enabled by GJ formation and the downstream signaling events in ECs that leads to SF1 secretion remain uncertain. We hypothesized involvement of Ca^{2+} in GJ-EC-TGF-β induced mural cell differentiation because of: i) its canonical involvement in stimulus secretion coupling in many cell types, including ECs[21,31], ii) its ability to permeate GJs,[13] and iii) the probable increase in $[Ca^{2+}]_i$ in ECs following formation of a GJ with MCs, due either to the higher basal $[Ca^{2+}]_i$ in MCs or to signal (e.g.: PDGF)-generated gradients across the GJ. Thus, the goal of the current study was to determine whether an increase in $[Ca^{2+}]_i$ in ECs might be sufficient, without additional signals that might occur as a consequence of GJ formation and function, to induce ECs to release SF1 (to condition the bathing medium) to activate TGF-β, which would then stimulate MC differentiation towards a mural cell phenotype. Our results suggest that transient elevation of $[Ca^{2+}]_i$ in ECs with ionomycin did not substitute for EC-MC GJ formation with regard to TGF-β activation. However, $[Ca^{2+}]_i$ elevation did lead to medium conditioning (SF2 release, **Figure 6**) that elicited a less robust activation in naïve $MC^{-/-}$s of R-Smad signaling and differentiation. Like SF1, activation of this complementary pathway and the resulting conditioned medium (SF2) lead to MC differentiation through activation of activin like 4, 5 or 7 receptors (likely ALK7).

Despite well established roles for GJ communication and TGF-β signaling, interactions between ECs and MCs during vascular development and repair are admittedly complex and involve multiple signaling pathways that may exhibit a high degree of temporal and spatial specificity[12]. For example, it was recently reported that a gap junction mediated transfer of serotonin was necessary for pulmonary endothelial cell-induced, TGF-β-mediated, maintenance of contractile phenotype in pulmonary smooth muscle cells[7]. However, another report suggested that endothelial progenitor cell-induced differentiation of human mesenchymal stem cells toward a mural cell phenotype was mediated by MAPK-dependent and gap junction communication-independent mechanisms (although connexin expression was still present)[9]. These examples highlight that despite the seemingly conserved ability of endothelial cells to induce a differentiated phenotype in a number of precursor and lineage committed smooth muscle cells, there is also a high degree of context specificity with regard to the exact signaling mechanisms. Thus, mechanistic data from numerous contexts must be gathered such that signaling “bottlenecks” can be identified and exploited as therapeutic targets. Of the potential signaling pathways involved, those mediated by Ca^{2+} are particularly difficult to study because of the broad array of functions mediated by Ca^{2+}, the many mechanisms controlling its availability, and cell-type specific

differences in both these parameters. Ca^{2+}-stimulated secretion, whether by voltage-dependent or receptor-mediated mechanisms,[14,31] usually occurs in response to an initial rapid elevation of cytosolic $[Ca^{2+}]_i$ that is followed by a somewhat slower reduction to a sustained level that is above the basal level.[31] Peak $[Ca^{2+}]_i$ levels are typically sufficient to activate numerous signaling molecules (e.g., protein kinase C, calmodulin and numerous calmodulin-dependent kinases and phosphatases) and the sustained levels are often sufficient to maintain activation of these signaling molecules for extended periods[31]. Our data show that ionomycin treatment produces this general pattern of $[Ca^{2+}]_i$ elevation in both the ECs and $MC^{-/-}$s; however, this pattern of Ca^{2+} elevation did not induce the ECs to release the SF able to activate TGF-β. Thus, although we acknowledge that the pattern of $[Ca^{2+}]_i$ elevation produced by ionomycin may not fully mimic the signaling events in ECs triggered by MC-EC GJs, we find it unlikely that transient (global) elevation of $[Ca^{2+}]_i$ is the only signal in the ECs resulting from EC-MC GJ function to achieve TGF-β activation, in other words Ca^{2+} elevation in the ECs is unlikely to be sufficient to trigger release of the TGF-β activating SF. We do not, however, dismiss the possibility that Ca^{2+} signaling may be critical (necessary) for release of the TGF-β activating SF and for subsequent differentiation of MCs to occur. Neither do we dismiss the results of recent investigations of Ca^{2+} signaling in smooth muscle cells that have revealed many intricate mechanisms of spatial and temporal Ca^{2+} signaling (reviewed at: [2]), some of which may be germane to GJ-dependent, EC-induced, MC differentiation. An extensive body of work will be necessary to resolve this issue.

Signaling through members of the TGF-β superfamily is involved in many physiological and pathological processes; not surprisingly, activation and exact mechanisms of TGF- β action are context and cell type specific. Nevertheless, a clear role for TGF-β in mediating EC-induced upregulation of mural cell markers is well documented; canonically TGF-β action in that capacity is thought to be mediated by ALK5 receptors and downstream R-Smad signaling. While it has been reported that upon GJ formation between EC and MC TGF-β is activated by a SF secreted by ECs (SF1 in Figure 6), the signaling events leading to the secretion of SF1, as well as its identity, have not yet been determined. In the current study, ECs did not release a TGF- β activating SF in response to $[Ca^{2+}]_i$ elevation, but did condition the medium (release of an unidentified SF, SF2 in Figure 6) such that it stimulated $MC^{-/-}$ differentiation through activation of TGF- β type 1 receptors blocked by SB431542, (ALK4, 5, or 7). Although the conditioning stimulated by ionomycin (identity of SF2) is unknown, the ligands for the ALK4, 5, 7 receptor complexes are members of the TGF- β superfamily, which include activins and nodals. Most members of the TGF- β superfamily are secreted in latent forms that require activation.[10,19]The bioavailability and/or activation of TGF- β family members is impacted by alterations of physicochemical factors (e.g., pH, irradiation, reactive oxygen species, temperature changes), proteases (e.g., MMPs, plasmin, thrombin, calpain), heparanases, and regulators such as thrombospondin and integrin[19]. Thus, it is currently unclear whether SF2 is a member of the TGF-β superfamily or an

activator thereof, but our data suggest that SF2 is not MMP 1, 2, 3, 8 or 9. In addition, the presence of reverse transcription dependent PCR amplicons generated with primers for ALK 4 and ALK7 sequences expands the list of agents that might comprise SF2. ALK4 and 7 receptors are targeted by the activins, ALK4 is readily activated by both activin A and activin B, whereas ALK7 is selectively activated by activin B or activin AB. [1,29,30]That $MC^{-/-}$s up-regulate the expression of mural cell markers in response to activin B but not activin A suggests that SF2 is either active ligand for ALK7 (eg.: activin B, activin AB, or nodal) or a factor able to release such a ligand from matrix binding sites (possibly, like activin A, proteoglycan component of the matrix[23]. Since transcripts for both ALK4 and ALK7 were detected in $MC^{-/-}$s and since ALK4 is the preferred binding partner of both activins, further experiments will be needed to determine whether the presence of ALK7 alone is both necessary and sufficient for the ionomycin-EC-induced $MC^{-/-}$ differentiation reported here. Nonetheless, these data are the first to implicate ALK receptor signaling dependent ligands, other than TGF-β, in the EC-induced mural cell differentiation required for vessel formation. In the context of existing literature, our data suggest a novel pathway that warrants further exploration and support the increasingly recognized notion that despite the limited number of receptors and mediators, TGF-β signaling is complex due to numerous modes of activation and regulation by a large array of complementary pathways.

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Disclosures: none

Figure Legends

Figure 1: Effect of ionomycin and co-culture with MCs on EC $[Ca^{2+}]_i$. **A)** Ionomycin stimulates a transient increase in $[Ca^{2+}]_i$ in ECs (n=39), Re43 (n=11) and $MC^{-/-}$s (n=33) that returns to somewhat elevated but stable levels within minutes. **B)** Basal EC $[Ca^{2+}]_i$ was unaffected by continuous ionomycin exposure for 24 hours or co-culture (24h) with either Re43 or $MC^{-/-}$s.

Figure 2: SMA expression by $MC^{-/-}$s is increased in an ionomycin-EC dependent fashion. **A**) Direct Treatment and Conditioned Medium protocols. **B**) Representative Western blots showing SMA expression in $MC^{-/-}$s exposed directly to TGF-β or ionomycin, and ECs or EC:$MC^{-/-}$ co-cultures exposed directly to ionomycin, each with its paired vehicle treated control. Note that ECs do not express detectable levels of SMA. **C**) Representative Western blots of SMA expression by naïve $MC^{-/-}$s exposed for 48h to medium conditioned by $MC^{-/-}$s, EC:$MC^{-/-}$ co-cultures, or ECs, exposed (+) or not (-) to ionomycin. **D & E**) Summary of density differences between treated and parallel vehicle treated controls across multiple experiments. **D**) SMA expression by $MC^{-/-}$s is increased when directly stimulated with TGF-β or with ionomycin when ECs are present,

but not by ionomycin when ECs are absent. **E**) Naïve $MC^{-/-}$s are stimulated to increase SMA expression by medium conditioned by ionomycin stimulated ECs or EC:MC co-culture. * indicates significant increase relative to paired, vehicle treated controls. Sample sizes indicated parenthetically.

Figure 3: TGF-β and MMPs are not necessary, but Smad2/3 signaling via ALK4, 5 and/or 7 is necessary for ionomycin-EC-induced differentiation of naïve $MC^{-/-}$s. (**A**) TGF-β neutralizing antibody did not prevent the increase in SMA expression by $MC^{-/-}$s exposed to medium conditioned by ionomycin treated ECs, but did prevent a TGF-β-induced increase. (**B**) Inhibition of MMPs with GM-6001 during conditioning of medium by ECs did not prevent the stimulatory impact of that medium on SMA expression by naïve $MC^{-/-}$s. (**C**) ALK4, 5 & 7 receptor inhibition with SB-431542 prevented the increase in SMA expression induced by ionomycin-EC conditioned medium. *Indicates significant increase in SMA expression relative to vehicle treated controls. Sample sizes indicated parenthetically.

Figure 4: Ionomycin-EC conditioned medium stimulates, possibly through ALK4 or ALK7 receptors, phosphorylation of Smad2/3 in $MC^{-/-}$s. **A**) Direct stimulation of $MC^{-/-}$s with exogenous TGF-β leads to Smad2/3 activation. **B**) Medium conditioned by ionomycin treated ECs modestly increased pSmad3 but not pSmad2 levels. *Indicates significant increase in pSmad expression relative to untreated controls. Sample sizes indicated parenthetically. RT-PCR reveals both ALK4 (**C**) and ALK7 (**D**) expression by $MC^{-/-}$s. In (C) Lanes 1&10 contain ladder; in (D) Lane 1 contains ladder, Lane 2 is empty. In both cases only primer set 1 was effective in generating RT-PCR amplicons.

Figure 5: Activin B, but not Activin A, stimulates a dose-dependent increase in SMA expression by $MC^{-/-}$s. * Indicates significant increase in SMA expression, n=5 for both ligands.

Figure 6: Proposed conceptual model for mechanisms contributing to EC-induced MC differentiation. ECs secrete PDGF-B, which attracts MCs that form junctions upon contact. These junctions are composed, in part, of Cx43-comprised GJs. GJ formation stimulates the EC to release soluble factor 1 (SF-1), which activates TGF-β. TGF-β activates ALK5 and Smad2/3 signaling. A second soluble factor (SF-2) may also be released by the ECs upon GJ formation, and is certainly released when $[Ca^{2+}]_i$ increases in response to ionomycin. SF2 also activates Smad2/3 signaling most likely through ALK7 (though contribution from ALK5 cannot be excluded).

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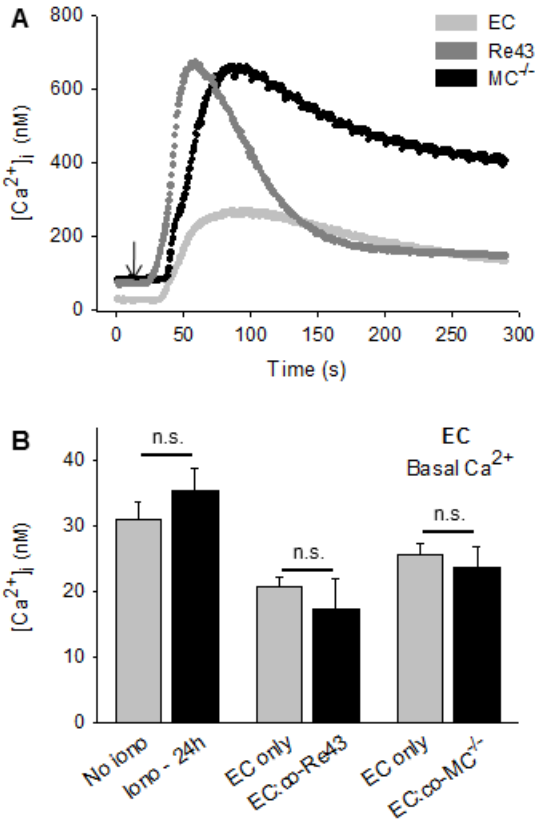
Figure 1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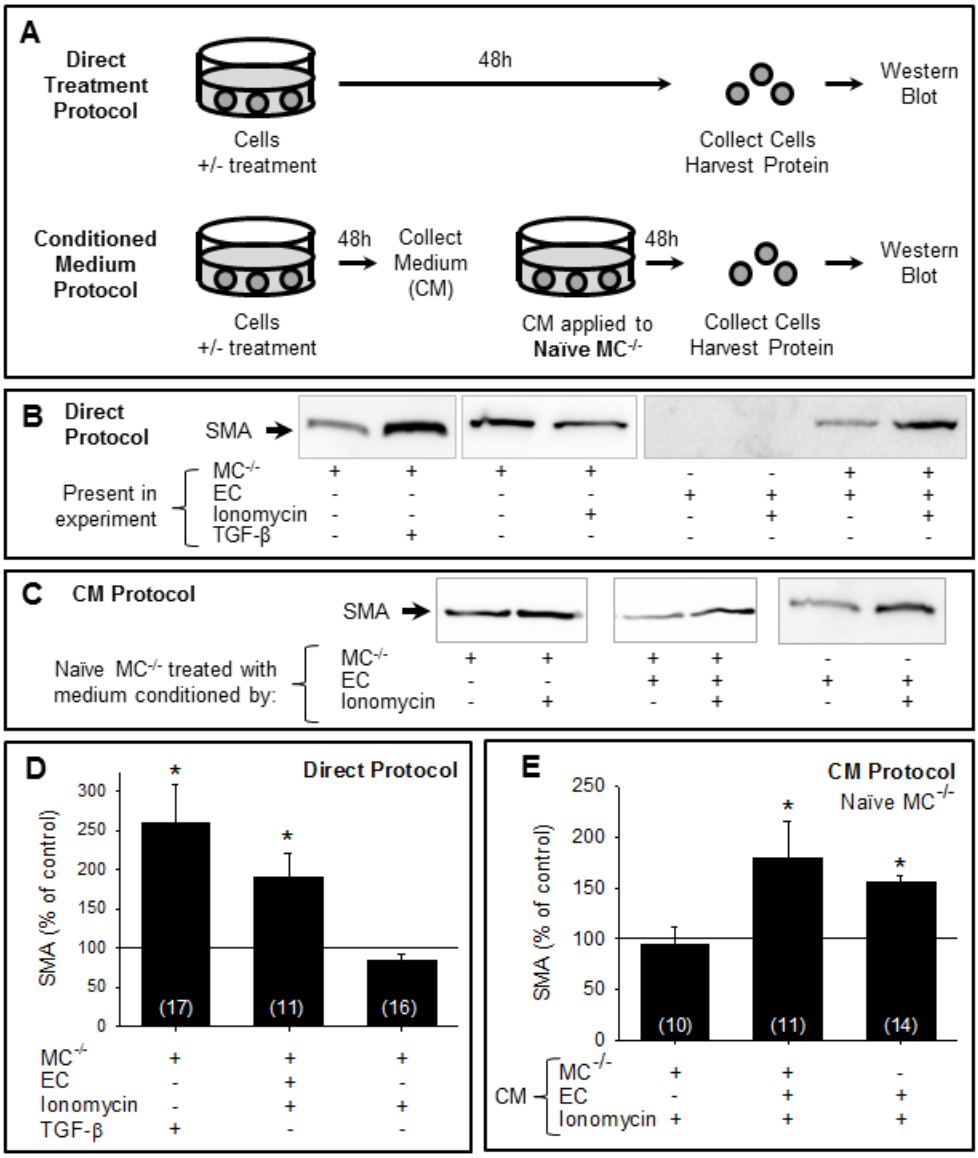
800
600
400
200
0
[Ca2+]i (nM)
0
50
100
150
200
250
300
Time (s)
EC
Re43
MC-/-
B
40
30
20
10
0
[Ca2+]i (nM)
n.s.
n.s.
n.s.
EC
Basal Ca2+
No iono
Iono - 24h
EC only
EC:co-Re43
EC only
EC:co-MC-/-

Figure 2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Direct Treatment Protocol
48h
Western Blot
Cells +/- treatment
Collect Cells Harvest Protein
Conditioned Medium Protocol
48h
Collect Medium (CM)
48h
Western Blot
Cells +/- treatment
CM applied to Naïve MC-/-
Collect Cells Harvest Protein
B
Direct Protocol
SMA
Present in experiment
MC-/-
EC
Ionomycin
TGF-β
C
CM Protocol
SMA
Naïve MC-/- treated with medium conditioned by:
MC-/-
EC
Ionomycin
D
Direct Protocol
SMA (% of control)
(17)
(11)
(16)
MC-/-
EC
Ionomycin
TGF-β
E
CM Protocol
Naïve MC-/-
SMA (% of control)
(10)
(11)
(14)
CM
MC-/-
EC
Ionomycin

Figure 3

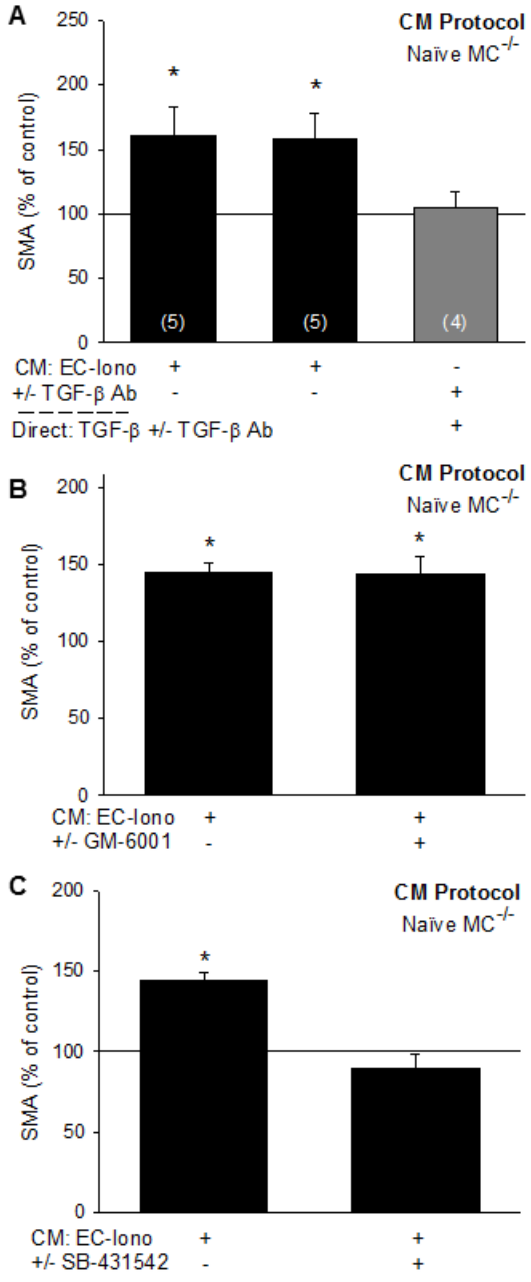
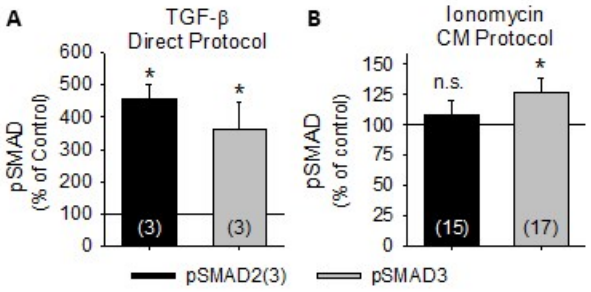
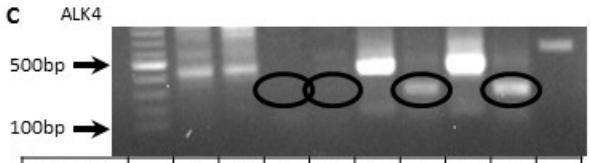

A
CM Protocol
Naïve MC-/-
SMA (% of control)
250
200
150
100
50
0
*
*
(5)
(5)
(4)
CM: EC-Iono + + -
+/- TGF-β Ab - - +
Direct: TGF-β +/- TGF-β Ab +
B
CM Protocol
Naïve MC-/-
SMA (% of control)
200
150
100
50
0
*
*
CM: EC-Iono + +
+/- GM-6001 - +
C
CM Protocol
Naïve MC-/-
SMA (% of control)
200
150
100
50
0
*
CM: EC-Iono + +
+/- SB-431542 - +

Figure 4





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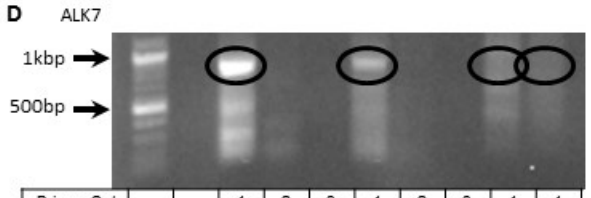
RT	-	-	+	-	+	+	+	+	+	-



Primer Set	-	-	1	2	3	1	2	3	1	1
Template	-	-	MC	MC	MC	Min	Min	Min	-	MC
RT	-	-	+	+	+	+	+	+	+	-

Figure 5

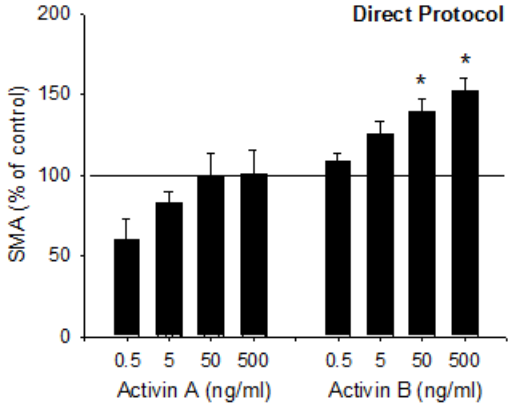
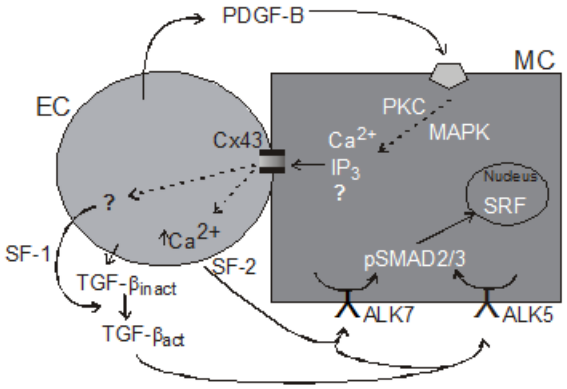

Direct Protocol
SMA (% of control)
200
150
100
50
0
*
*
0.5
5
50
500
Activin A (ng/ml)
0.5
5
50
500
Activin B (ng/ml)

Figure 6


PDGF-B
MC
EC
PKC
MAPK
Ca2+
Cx43
IP3
?
Nucleus
SRF
?
↑Ca2+
SF-1
SF-2
pSMAD2/3
TGF-βinact
TGF-βact
ALK7
ALK5

Graphical Abstract